α2,6-Sialylation promotes binding of placental protein 14 via its Ca²⁺-dependent lectin activity: insights into differential effects on CD45RO and CD45RA T cells

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Placental protein 14 (PP14; glycodelin) is a pregnancy-associated immunoregulatory protein that is known to inhibit T cells via T-cell receptor desensitization. The recent demonstration of PP14 as lectin has provided insight into how it may mediate its CD45 glycoprotein-dependent T-cell inhibition. In this study, we have investigated PP14's lectin-binding properties in detail. Significantly, PP14 reacts with *N*-acetyllactosamine (LacNAc) as was also found for members of the galectin family, such as the potent immunoregulatory protein, galectin-1. However, in contrast to galectin-1, PP14's binding is significantly enhanced by α2,6-sialylation and also by the presence of cations. This was demonstrated by preferential binding to fetuin as compared with its desialylated variant asialofetuin (ASF) and by using free α2,6- versus α2,3-sialylated forms of LacNAc in competitive inhibition and direct solid-phase binding assays. Interestingly, from immunological point of view, PP14 also binds differentially to CD45 isoforms known to differ in their degree of sialylation. PP14 preferentially inhibits CD45RA⁺, as compared with CD45RO⁺ T cells, and preferentially co-capped this variant CD45 on the T-cell surface. Finally, we demonstrate that PP14 promotes CD45 dimerization and clustering, a phenomenon that may regulate CD45 activity.

Key words: CD45/galectin/glycodelin/sialic acid/T cell

Introduction

Placental protein 14 (PP14; glycodelin) is a major secreted glycoprotein of pregnancy that is abundant in amniotic fluid (AF) and in first-trimester serum of pregnant women (Julkunen *et al.*, 1985, 1986). PP14 is one of a relatively limited set of immunoregulatory proteins known to affect T cells directly by targeting an early step of T-cell activation (Rachmilewitz *et al.*, 1999). PP14's T-cell inhibitory activity

depends upon its recruitment to the antigen-presenting cells (APC): T-cell contact site and accessing the triggered TCR (Rachmilewitz *et al.*, 2002). Consequently, PP14 mediates its anti-inflammatory activity by elevating the T-cell activation threshold thereby rendering T cells less sensitive to activation (Rachmilewitz *et al.*, 2001). Interestingly, PP14's activity leads to decreased stability of T cell receptor (TCR)-induced phosphorylated proteins (Rachmilewitz *et al.*, 2002), possibly because of increased activity or accessibility of phosphatases.

Therefore, in a recent study, we proposed a connection between PP14 and the tyrosine phosphatase receptor, CD45. In this study, the inhibitory effect of PP14 was demonstrated to be dependent on CD45 and possibly linked to PP14 lectin-binding capacity (Rachmilewitz *et al.*, 2003). Specifically, PP14's inhibitory effects are dependent on the surface expression of the extracellular and/or transmembrane domains of the tyrosine phosphatase receptor, CD45. Moreover, PP14 and CD45 co-capped with each other, pointing to their physical linkage. Perhaps most interestingly, we have determined that PP14 binds to T-cell surfaces in a carbohydrate-dependent fashion. Thus, while PP14 binds to CD45, one of the most abundant glycoproteins on T cells, it binds in a lectin-like mode to other T-cell surface glycoproteins as well. This fundamental insight into PP14's binding potentials casts its immunoregulatory activities in a new light, with similarities to CD22 and galectin-1, mammalian lectins that also bind to CD45. These proteins exploit sugar-encoded messages of CD45 glycans for binding and intracellular signaling (Gabius *et al.*, 2004; Villalobo *et al.*, in press).

There are growing numbers of immunoregulatory proteins with lectin properties, two of which have been shown to associate with CD45. In detail, CD22 (also termed siglec-2), is a member of the siglec family mainly expressed by cells of the hematopoietic system (Crocker and Varki, 2001). CD22's N-terminal V-set Ig domain binds α2,6-sialylated *N*-glycans-linked sialoglycoconjugates and is a specific B-cell receptor with features of inhibitory receptors. Using a soluble CD22 version in combination with anti-CD3 for cross-linking of CD3 on T-cell surfaces, it was shown that CD22 binds to CD45 and inhibits early T-cell activation events (Aruffo *et al.*, 1992). These effects are consistent with those observed upon colligation of CD3 and CD45 with antibody (Turka *et al.*, 1992). However, the biological significance of the association of CD22 with CD45 is controversial, given that these inhibitory effects required the extensive cross-linking by a the fusion protein bound to CD45 that is not likely to occur *in vivo*, particularly with a cell surface ligand (Zhang and Varki, 2004).

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Galectin-1 belongs to a family of endogenous growth/adhesion-regulatory lectins sharing Ca^{2+}-independent binding activity to β-galactosides and sequences/folding homology (Lopez-Lucendo *et al.*, 2004). Of note, galectin-1 is expressed by T cells and induces apoptosis of activated T cells probably through caspase activation (Goldstone and Lavin, 1991; Sturm *et al.*, 2004). Because of its lectin activity, galectin-1 can interact with distinct glycoproteins on the T-cell surface in addition to CD45 including CD2, CD3, CD4, CD7, CD43, and components of lipid rafts such as ganglioside GM1; however, CD45 seems important for their T-cell inhibitory function (Perillo *et al.*, 1995; Nguyen *et al.*, 2001; Siebert *et al.*, 2003). Underscoring its therapeutic potential, pioneering reports revealed that administration of galectin-1 prevented experimental induced autoimmune encephalomyelitis in rats (Offner *et al.*, 1990) and was prophylactic and therapeutic in a rabbit model of autoimmune myasthenia gravis (Levi *et al.*, 1983).

In this study, we have set out to explore PP14's lectin properties in depth, whereby differences in the lectin properties of PP14 and galectin-1 emerged. Specifically, we show that while PP14, like galectin-1, binds to *N*-acetyllactosamine (LacNAc; Galβ1,4GlcNAc) sequences, it differed from galectin in its preferential binding to α2,6-sialylated epitopes. Intriguingly, this binding specificities correlates with differential functional effects of PP14 on T-cell subsets differing in their CD45 surface glycoforms. Given PP14's preferential binding to α2,6-sialylated LacNAc (in contrast to the galectin) PP14 favors the CD45RA glycoform and promotes receptor clustering and dimerization.

Results

PP14's lectin binding is not dependent of its glycosylation

PP14 is N-glycosylated, and its glycosylation pattern is thought to influence its various functions (Morris *et al.*, 1996; Seppala *et al.*, 1998). Therefore, as a prelude to detailed analyses of PP14's properties as lectin, we first asked whether PP14's oligosaccharide side chains are essential for its ability to bind T-cell surface. In fact, presence of cell surface lectins can account for such binding. To this end, PP14·Fc$_{\gamma 1}$ was treated with PNGase F, a glycosidase that cleaves *N*-glycosyl groups in their entirety at the asparagine linkage, and the ability of this N-deglycosylated PP14 to bind to CD4$^+$ T cells was tested. The completeness of PP14 deglycosylation was documented by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 1, inset). As shown in Figure 1, the PNGase F treatment of PP14·Fc$_{\gamma 1}$ did not affect its ability to bind to T cells, as detected by immunofluorescence and flow cytometry, suggesting that PP14's own glycosylation is not required for its cell-binding ability.

Contribution of calcium ions and sialylation to PP14 activity as lectin

We have previously demonstrated that PP14 has lectin-binding properties, by showing that the glycoprotein asialofetuin (ASF) and to a lesser extent, lactose and cellobiose, competitively inhibit PP14's binding to T cells (Rachmilewitz
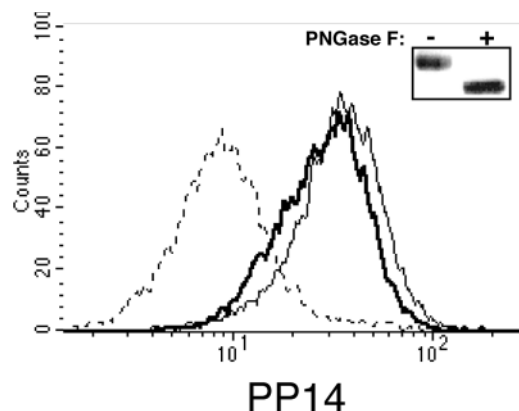

Fig. 1. PP14 binding to T-cell surface is independent of its glycosylation. PP14·Fc$_{\gamma 1}$ was either treated with or without PNGase F for 30 min at 37°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was used to assess efficiency of enzyme treatment (inset). Purified CD4$^+$ T cells were incubated with either PP14·Fc$_{\gamma 1}$ (thick line) or with PNGAse F treated PP14·Fc$_{\gamma 1}$ (thin line) for 30 min at 37°C. CTLA-4·Fc$_{\gamma 1}$ was used as a nonspecific Fc-receptor binding (dashed line). Similar results were obtained in three experiments.

et al., 2003). This was of particular interest given that another immunoregulatory protein functioning through CD45 is a lectin, namely galectin-1 (Perillo *et al.*, 1995; Pace *et al.*, 1999; Walzel *et al.*, 1999; Fouillit *et al.*, 2000; Nguyen *et al.*, 2001). We therefore decided to more thoroughly examine the carbohydrate specificity of PP14 and to compare these two immunomodulatory lectins in this respect. For this purpose, we started with solid-phase assays using two natural glycoproteins presenting bi- and/or triantennary *N*-glycans, which are potent binding partners for galectins.

We assessed direct binding of PP14·Fc$_{\gamma 1}$ to ASF in a solid-phase binding assay. Because activities of mammalian lectins in certain cases such as the C-type lectins or a P-type lectin is dependent on cations, especially Ca^{2+}, we initially surveyed binding activity in the presence of different cations. Assays were performed with ASF but also included the human pentraxin serum amyloid P component (SAP) as ligand for binding to PP14·Fc$_{\gamma 1}$ precoated onto wells. Many divalent cations influenced binding in this assay, and among them Ca^{2+}, Co^{2+}, Mn^{2+}, and Ba^{2+} yielding the most prominent effects (data not shown). We focused on Ca^{2+} for further analysis given its reactivity with C-type lectins and its physiological relevance. As shown in Figure 2A, both biotinylated ASF (left panel) and SAP (right panel) bound to PP14·Fc$_{\gamma 1}$ in a dose- and calcium-dependent fashion. Of note, this documentation of Ca^{2+} dependence separates PP14's activity from that of any galectin, which is invariably independent of Ca^{2+} ions.

In the given setting, PP14 had been immobilized to the plastic surface. To exclude an impact of surface binding we reconfigured the binding assay so that glycoproteins were instead the component prebound to wells in the solid-phase mimicking a cell surface, and binding of PP14·Fc$_{\gamma 1}$ was assayed. PP14·Fc$_{\gamma 1}$ bound to both ASF and its sialylated form fetuin in a calcium-dependent manner (Figure 2B). Interestingly, binding to fetuin was somewhat higher than
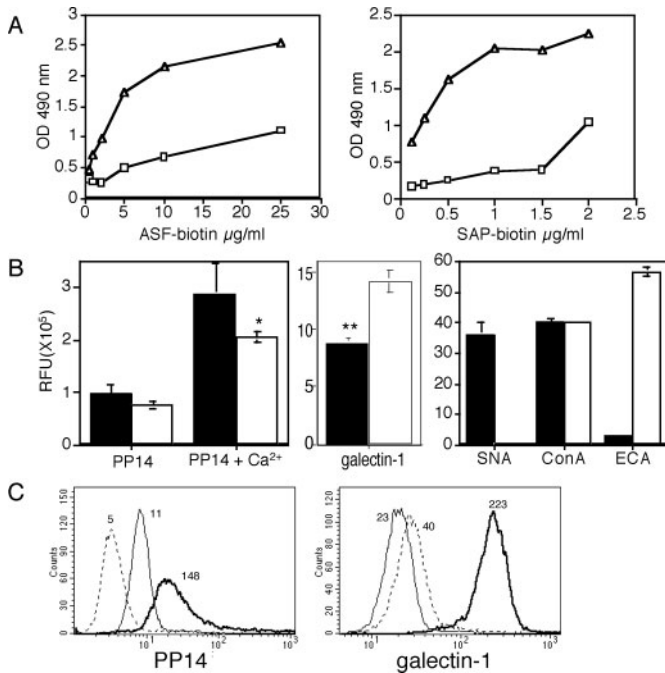
Fig. 2. Higher, Ca^{2+} dependent, binding of PP14 to fetuin as compared with its desialylated derivative, ASF. (**A**) The extent of binding at various concentrations of either ASF (**left** panel) or SAP (**right** panel) to immobilized PP14·Fc$_{\gamma l}$ was tested, as described in *Materials and methods*, in the presence (triangles) or absence (squares) of 10 mM Ca^{2+}. (**B**) The binding of either PP14·Fc$_{\gamma l}$ (**left** panel) or galectin-1 (**right** panel) to fetuin (black bars) and ASF (white bars) was tested, as described in *Materials and methods*. PP14·Fc$_{\gamma l}$ binding was carried out in the presence or absence of 10 mM Ca^{2+}. As control, the plant lectins, SNA, ConA, and ECA (all at 5 mg/mL) were used (**right** panel). The results represent the mean ± SD of quadruplicate wells. Similar results were obtained in two additional experiments. (**C**) Purified CD4$^+$ T cells were incubated with either PP14·Fc$_{\gamma l}$ (**left** panel) or galectin-1 (**middle** panel) for 30 min at 37°C (thick line). For purposes of competitive inhibition, cells were incubated with either fetuin (dotted lines) or ASF (thin lines; 0.5 mg/mL), before the addition of the lectins. Cell preparation and labeling was as described in *Materials and methods*. Numbers indicate the mean fluorescence intensities of each histogram. The results shown are representative of four different experiments. *, $p < 0.05$ and ** $p < 0.0001$.

to its desialylated derivative (Figure 2B, left panel). This contrasts with the behavior of galectin-1, with its preferential binding to ASF (Figure 2, middle panel). The validity of this solid-phase direct binding assay was verified using plant lectins, specifically by demonstrating that *Sambucus nigra* agglutinin (SNA) (with selectivity for α2,6-linked sialylgalactose) binds solely to fetuin, *Erytherina cristagalli* agglutinin (ECA) (which recognizes nonsialylated terminal LacNAc) binds preferentially to ASF, and ConA (with selectivity for the trimannoside core part of *N*-glycans) binds equally to both (Figure 2B, right panel). Evidently, sialylation is at least tolerated by PP14.

To complement these direct binding assays, we next turned to analysis of PP14 binding to intact cells, with a goal of further probing the role of sialylation that emerged from the direct binding analyses (Figure 2B). Specifically, we compared the capacities of fetuin versus ASF to competitively inhibit PP14·Fc$_{\gamma l}$ binding to CD4$^+$ T cells, as monitored by immunofluorescence and flow cytometry. At

an intermediate concentration (0.2–0.5 mg/mL), ASF demonstrated less competitive inhibitory capacity than its sialylated form (Figure 2C, left panel). By contrast, and as expected for an established β-galactoside-binding lectin, galectin-1's binding to T cells was more effectively blocked by ASF (Figure 2C, right panel). This suggested that sialylation of oligosaccharide branches increases PP14, but not galectin-1, baseline binding to glycoprotein ligands.

### Role for α2,6-sialylation in PP14 binding

Next, we looked in more detail at the structural determinants of carbohydrate influencing PP14 binding, focusing on sialylation. We tested the ability of several free carbohydrates to competitively block PP14·Fc$_{\gamma l}$'s binding to CD4$^+$ T cells, using immunofluorescence and flow cytometry as readout (Figure 3A). Our choice of free carbohydrates was driven by the parallels to galectin-1, with an emphasis on sialylated derivatives. LacNAc, but not *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc) or lactose (Galβ1,4Glc; all at 60 mM), demonstrated moderate inhibition of PP14·Fc$_{\gamma l}$ binding to the T cells. Significantly, α2,6-linked sialic acid in LacNAc (even at only 28 mM) was effective and showed substantially more inhibition than LacNAc, whereas the α2,3-sialylated isomer of LacNAc had a markedly reduced competitive inhibitory capacity in this cell-binding assay.

To solidify this difference in α2,6- versus α2,3-linkage of sialic acid, we looked at binding to neoglycoconjugates in a solid-phase binding assay. These probes present a homogeneous sugar population as ligand, allowing unambiguous conclusions. More PP14·Fc$_{\gamma l}$ bound to immobilized neoglycoconjugate harboring SAα2,6Galβ1,4GlcNAc- as opposed to the polymer with the α2,3-isomer. This difference was evident at each of two tested concentrations (Figure 3B). Taken together, these findings strongly suggest that α2,6-sialylated glycoforms may have a preferential effect on PP14 lectin binding, in contrast to the deleterious effect of this type of sialylation on galectin-1 binding.

### Role for sialylation in PP14-mediated T-cell inhibition

To complement the cell-based competitive inhibition and solid-phase binding assays, we next determined the implications of sialylation on T-cell surfaces for PP14 binding and function. We have used *Vibrio cholerae* sialidase which preferentially hydrolyzes α2,3-linkages of sialic acid and also acts on α2,6 and α2,8-linkages (Corfield *et al.*, 1983). CD4$^+$ T cells were treated with this sialidase, and PP14·Fc$_{\gamma l}$ binding was assessed by immunofluorescence and flow cytometry. The extent of desialylation was determined using fluorescein isothiocyanate (FITC)-labeled SNA as probe. This control established that the sialidase was active, with partial desialylation evident (Figure 4A, left panel). Even this level of desialylation was sufficient to yield an observable effect on PP14 binding (Figure 4A, right panel).

Having demonstrated a contribution of sialylation to PP14 lectin binding, we next addressed its functional consequences. T cells stimulated with the polyclonal activator Staphylococcal Enterotoxin B (SEB) secrete high levels of interferon-γ (IFN-γ), a process that is inhibited by PP14 (Mishan-Eisenberg *et al.*, 2004). Therefore, the inhibitory
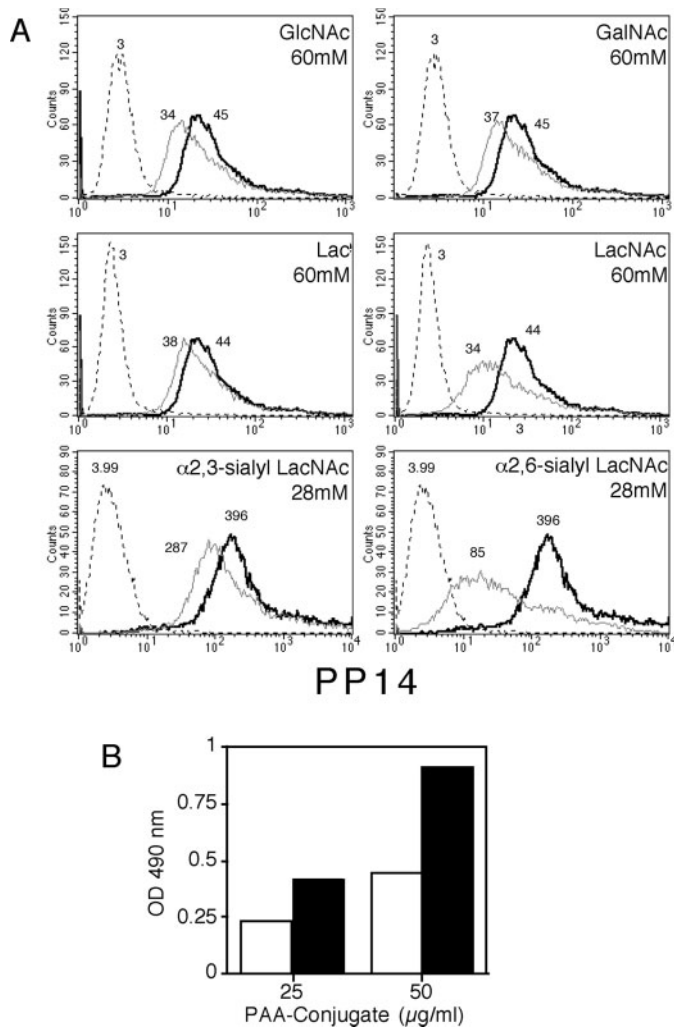
Fig. 3. Preferential binding of PP14 to α2,6-sialylated *N*-acetyllctosamine. (A) PP14·Fc$_{\gamma 1}$ binding to T cells can be competitively inhibited with oligosaccharides. Purified CD4$^+$ T cells were incubated with PP14·Fc$_{\gamma 1}$ for 30 min at 37°C. For purposes of competitive inhibition, *N*-acetylglucosamine (GlcNAc, 60 mM), *N*-acetylgalactosamine (GalNAc, 60 mM), lactose (60 mM), LacNAc (60 mM), α2,3-sialyl LacNAc (28 mM) or α2,6-sialyl LacNAc (28 mM), and ASF (1 mg/mL) were added before the addition of PP14·Fc$_{\gamma 1}$. Cell preparation and labeling was as described in Figure 2. Thick line, PP14·Fc$_{\gamma 1}$ binding; thin line, PP14·Fc$_{\gamma 1}$ binding in the presence of the competitive inhibitors; and dotted line, PP14·Fc$_{\gamma 1}$ binding in the presence of ASF (1 mg/mL) that represents maximal competition. Numbers indicate the mean fluorescence intensities of each histogram. The results shown are representative of four different experiments. (B) PP14·Fc$_{\gamma 1}$ binding to either α2,3-sialylLacNAc or α2,6-sialylLacNAc-bearing neoglycoconjugates. PP14·Fc$_{\gamma 1}$ was immobilized onto the surface of microtiter plates, and either α2,3-sialylLacNAc (white bars) or α2,6-sialylLacNAc (black bars) covalently linked to a biotinylated PAA were added to the wells at the indicated concentrations in the presence of 10 mM Ca^{2+}. The level of binding was measured as described in *Materials and methods* and is expressed as the optical density at 490 nm. No binding of control PAA to PP14 was detected. Comparable results were obtained in three experiments.

effect of PP14 on IFN-γ secretion from SEB-stimulated CD4$^+$ T cells, with or without prior sialidase treatment, was assessed. Using AF as a rich source of natural PP14, we found marked loss of PP14-mediated inhibition after desialylation, at varying concentrations of AF (Figure 4B, left
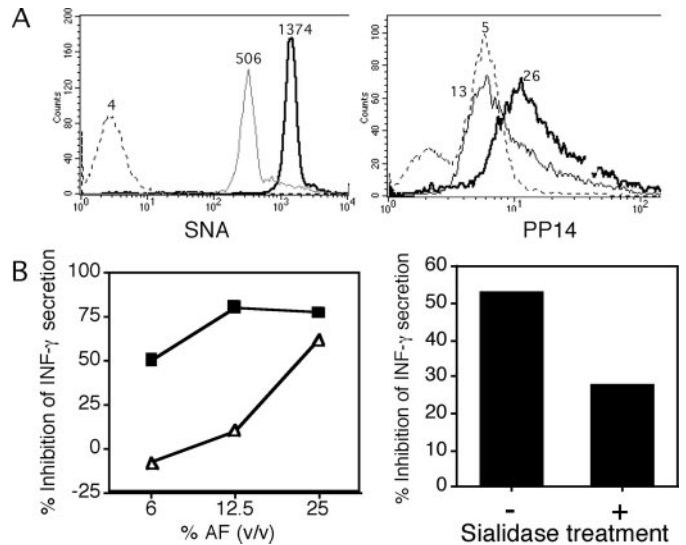

Fig. 4. Sialidase treatment of CD4$^+$ T cell reduces their sensitivity to PP14-mediated inhibition. Purified CD4$^+$ T cells were incubated with or without sialidase and then washed. (A) The levels of cell surface sialylation before (black line) and after (grey line) sialidase treatment were verified with FITC-conjugated SNA (**left** panel); the dashed line represent control unstained cells. Either sialidase-treated (grey line) or control (black line) CD4$^+$ T cells were incubated with PP14·Fc$_{\gamma 1}$ for 30 min at 37°C and were then processed for detecting bound PP14·Fc$_{\gamma 1}$, as described in *Materials and methods* (**right** panel); the dashed line represents CTLA-4·Fc$_{\gamma 1}$ binding that was used as a negative control for nonspecific Fc-receptor binding. Numbers indicate the mean fluorescence intensities of each histogram. (B) Either sialidase-treated (open triangles) or control (filled squares) CD4$^+$ T cells were stimulated with SEB (1 ng/mL) and monocytes as APC in the presence or absence of either AF (**left** panel) or PP14·Fc$_{\gamma 1}$ (100 mg/mL; **right** panel). After 16 h, the conditioned media were collected, and the level of IFN-g was determined by enzyme-linked immunoadsorbent assay (ELISA). SEB-stimulated sialidase-treated T cells secreted 0.85 ng/mL IFN-g, and untreated T cells secreted 0.95 ng/mL IFN-g. The data are presented as percentage of inhibition of IFN-g secretion. The data in **A** and **B** were confirmed in three independent experiments.

panel). The AF result was confirmed using PP14·Fc$_{\gamma 1}$, which again yielded substantially reduced inhibition of IFN-g secretion for sialidase-treated T cells (Figure 4B, right panel).

Differential effects of PP14 on CD45RA versus CD45RO T-cell subsets

We next interrogated the biological and functional significance of PP14's observed carbohydrate-binding specificity. Interestingly, the alternatively spliced CD45RA and CD45RO isoforms, which define the naïve and memory/activated CD4$^+$ T-cell subsets, respectively, are known to differ with respect to their glycosylation and their resultant size, shape, and negative charge (Trowbridge and Thomas, 1994). Since we had previously shown that PP14's inhibitory capacity on T cells depends upon the presence of the extracellular domain of CD45 (Rachmilewitz *et al.*, 2003), it became of interest to ask whether CD45RA and CD45RO CD4$^+$ T cells differ in their sensitivity to PP14. CD45RA and CD45RO cells were enriched through both positive and negative magnetic antibody-microbead selection (Figure 5A), and the separate enriched subpopulations were stimulated with the polyclonal activator SEB in the presence of purified CD14$^+$ monocytes as APC. Both AF
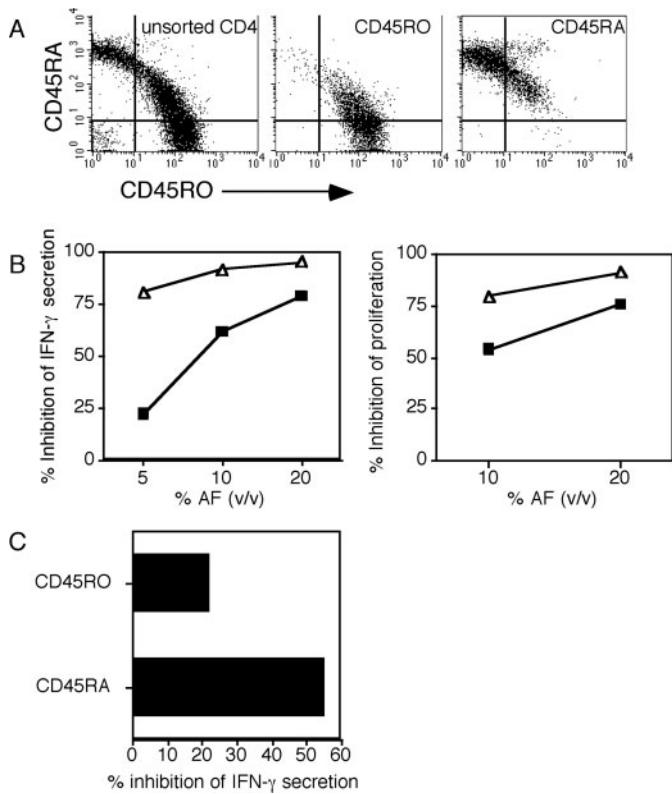
Fig. 5. Higher sensitivity of CD45RA T cells to PP14-mediated inhibition, as compared with CD45RO T cells. (**A**) CD4^{+} T cells separated into CD45RA and CD45RO subsets using the appropriate magnetic microbeads. The levels of cell surface CD45 isoform expression on unsorted and sorted cells were verified with FITC-conjugated anti-CD45RO and PE-conjugated anti-CD45RA immunostaining. Cells were prepared and labeled, as described in *Materials and methods*, and 2×10^4 cells were analyzed by flow cytometry. (**B**) Either CD45RA (triangles) or CD45RO (squares) cells were activated for 72 h with monocytes and SEB (1 ng/mL) in the presence or absence of the indicated concentrations of AF, and either IFN-γ secretion (**left** panel) or cell proliferation (**right** panel) was tested. (**C**) CD45RA or CD45RO T cells were activated as in **B** in the presence or absence of PP14·Fc$_{\gamma l}$ (100 mg/mL), and the level of secreted IFN-γ was tested. The data in **B** and **C** are presented as percentage of inhibition. SEB-stimulated CD45RA and CD45RO T cells secreted 13.9 and 14.7 ng/mL IFN-γ, respectively. The results shown are representative of three separate experiments.

(Figure 5B) and PP14·Fc$_{\gamma l}$ (Figure 5C) preferentially inhibited IFN-γ secretion by (Figure 5B, left panel; Figure 5C) and cell proliferation of (Figure 5B, right panel) CD45RA CD4^{+} T cells, with CD45RO CD4^{+} T cells displaying less sensitivity to this inhibitor.

Since the total surface staining by labeled SNA (Figure 6A, left panel) and the binding *per se* of PP14 to both T-cell subsets (as expected for a lectin binding to carbohydrate determinants common to a series of glycoproteins) (Figure 6A, right panel) as well as the affect of sialidase treatment on both SNA staining and PP14 binding (data not shown) was indistinguishable by immunofluorescence and flow cytometry, we attempted to address this emerging issue by looking for differences in PP14 : CD45 clustering at the cell surface. We previously demonstrated co-capping of PP14·Fc$_{\gamma l}$ and CD45 when the former is cross-linked using anti-Fc$_{\gamma l}$ mAb, suggesting physical interaction between the

two (Rachmilewitz *et al*., 2003). This experimental approach was now repeated, this time on cells separated on the basis of their CD45 isoform expression. There was a small but significantly increased extent of co-capping observed for the CD45RA as opposed to the CD45RO isoforms (Figure 6B). This difference was clearly evident when both the number of capped cells with associated CD45 and the average area of PP14 : CD45 co-capping (yellow) of the total capped PP14·Fc$_{\gamma l}$ (red) per cell were calculated (Figure 6C). As negative control, cross-linking of bound CTLA-4 Fc$_{\gamma l}$ with anti-Fc$_{\gamma l}$ mAb showed no co-capping, exactly as we previously demonstrated (data not shown; Rachmilewitz *et al*., 2003). Taken together, these findings demonstrate a difference between CD45RA (naïve) and CD45RO (memory/activated) T-cell subsets in their susceptibility to PP14 inhibition, which may result from differential physical interactions (direct or indirect) between PP14 and the two CD45 variants expressed on these cellular subsets.

Promotion of CD45 dimerization and clustering by PP14

Given the CD45-dependence of PP14-mediated T-cell inhibition (Rachmilewitz *et al*., 2003), along with the present data suggesting differential effects of PP14 on CD45RA versus CD45RO CD4^{+} T cells, we now asked whether this effect is somehow tied to CD45 dimerization. Previous data had shown differential homodimerization of CD45 on T-cell subsets expressing the variants, with greater homodimerization for the CD45RO isoform, presumably due to reduced glycosylation and, in particular, sialylation (Xu and Weiss, 2002). Here we similarly assessed CD45 dimerization potential, in this case looking for PP14 effects. As observed in previous study (Xu and Weiss, 2002), we also found more dimerization for CD45RO, as compared with CD45RA (Figure 7). However, a relatively wide band was observed in our results, thus the shift to higher molecular weight is probably not only due to dimerization *per se* but also the result of CD45 clustering with other associated proteins. Association of CD45 with other cell surface proteins is well documented (reviewed in Altin and Sloan, 1997). Significantly, PP14·Fc$_{\gamma l}$ as well as AF substantially increased the dimer/high molecular weight to monomer ratio for both CD45RA and CD45RO (Figure 7). Our data therefore suggest that PP14 promotes CD45 dimerization and/or association with other cell surface proteins.

Discussion

PP14 is a major protein of pregnancy with T-cell immuno-regulatory properties (Rachmilewitz *et al*., 1999, 2001, 2002; Mishan-Eisenberg *et al*., 2004). In exploring this at a mechanistic level, we have previously implicated PP14 binding to surface glycoproteins, and in particular the tyrosine phosphatase receptor CD45, the expression of which is essential for PP14 inhibitory activity (Rachmilewitz *et al*., 2003). This study has also suggested that PP14 interacts with the T-cell surface through carbohydrate recognition given that ASF and to a lesser extent lactose can competitively compete with PP14 binding (Rachmilewitz *et al*., 2003), just as in the case of galectin-1. This parallel between PP14 and galectin-1, another lectin with immunomodulatory
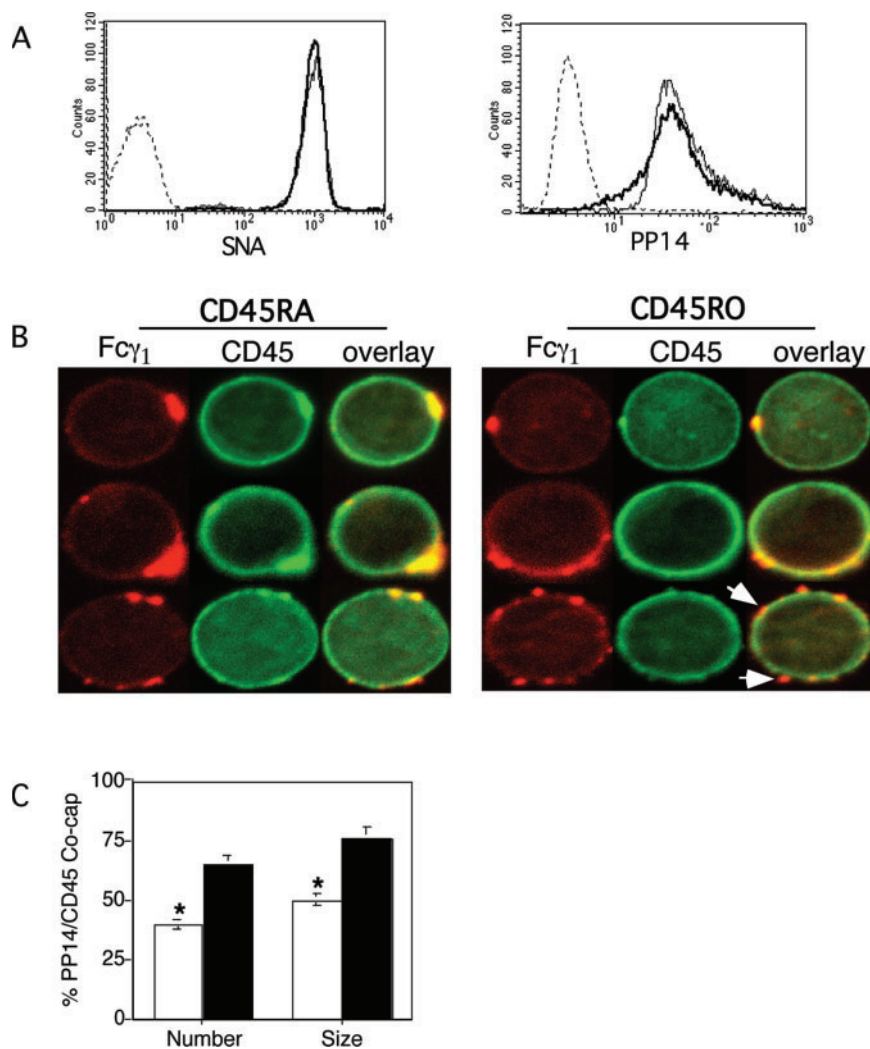
Fig. 6. PP14 preferentially co-caps with CD45RA as compared with the CD45RO isoform. (**A**) Both SNA and PP14·Fc$_{\gamma1}$ bind equally well to CD45RA and CD45RO T-cell surfaces. Purified CD45RA (black line) or CD45RO (grey line) CD4$^+$ T cells were stained with SNA (**left** panel; dashed line, control unstained cells) or incubated with either PP14·Fc$_{\gamma1}$ or CTLA-4·Fc$_{\gamma1}$ (for nonspecific Fc-receptor binding; dashed lines—**right** panel) for 30 min at 37°C. Cells were prepared and labeled, as described in *Materials and methods*, and 1×10^4 cells were analyzed by flow cytometry to detect bound protein. (**B**) PP14·Fc$_{\gamma1}$ was incubated with either purified CD45RA (**left** panel) or CD45RO (**right** panel) CD4$^+$ T cells, and it was then induced to cap using anti-human IgG for cross-linking. Association with CD45 was demonstrated using a pan-specific anti-CD45 mAb. Dual color fluorescent analysis was performed with detection by confocal microscopy, with green representing CD45, red representing PP14, and yellow indicating areas of overlap. Four representative pseudocolor images for each cell type are shown. Arrows indicate PP14 aggregates without CD45 co-capping. (**C**) The level of PP14·Fc$_{\gamma1}$ and CD45 co-localization per cell was measured using Image Pro and is expressed as both the number CD45 : PP14 aggregates of the total number of PP14 aggregates and as the percentage area of co-localized PP14·Fc$_{\gamma1}$ and CD45 (yellow) relative to the total PP14·Fc$_{\gamma1}$ binding (red). The data represent an average of 118 CD45RO cells (white bars) and 108 CD45RA cells (black bars). Results are presented as mean ± SE; *, $p < 0.05$.

activities that acts through the CD45 receptor (Perillo *et al.*, 1995; Walzel *et al.*, 1999), provided the incentive for studying PP14's lectin-like properties and directed our choice of oligosaccharides that may serve as recognition motifs for PP14.

In this study, we have studied PP14's lectin properties. PP14 is highly glycosylated protein that is expressed as two gender-specific glycoforms in humans (Morris *et al.*, 1996). Significantly, these two glycoforms differ in their biological activities, as a result of changes in their glycosylation (Morris *et al.*, 1996; Mukhopadhyay *et al.*, 2004). The data presented in this study have shown that the oligosaccharide side chains are not essential for PP14's lectin-like activity, because it can bind equally well to T cells in its glycosylated

and deglycosylated form as well as when it was subjected to sialidase treatment (data not shown). Furthermore, we have demonstrated that PP14's activity as lectin is calcium-dependent, a property that distinguishes it from galectins.

The key finding in this study concerns the specific carbohydrate recognized by PP14. The data presented demonstrate that, like galectin-1, PP14's binding to glycoproteins is mediated by terminal LacNAc. However, the two lectins differ in the way they can handle presence of terminal α2,6-linked sialic acid. Whereas, galectin-1's binding to LacNAc sequence is abrogated in the presence of α2,6-linked sialic acid (Ahmad *et al.*, 2002; Amano *et al.*, 2003; Leppanen *et al.*, 2005), binding of PP14 is augmented by this core extension. Several findings support this conclusion. First
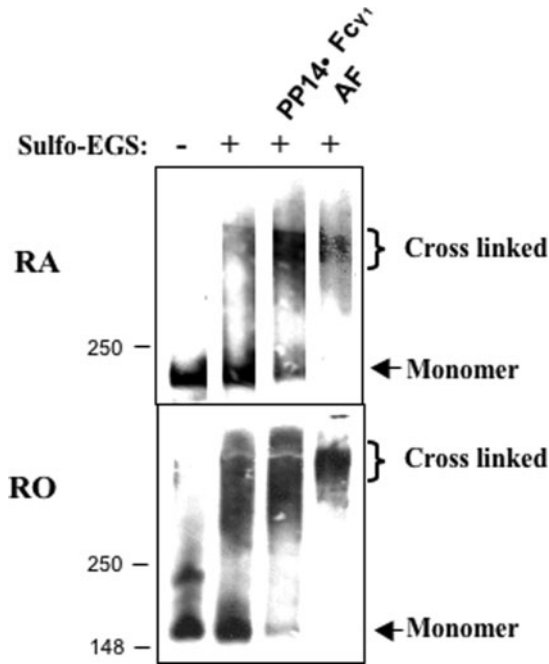
Fig. 7. PP14 promotes dimerization and clustering of CD45 in CD45RA and CD45RO T cells. Either CD45RA (**upper** panel) or CD45RO (**lower** panel) CD4^{+} T cells were incubated without or with either AF (10% v/v) or PP14·Fc$_{\gamma l}$ (30 µg/mL) for 30 min at 37°C. Cells were cross-linked with 2 mM Sulfo-EGS, and whole cell lysates were blotted with anti-CD45RA and anti-CD45RO mAb, respectively. The numbers on the left indicates molecular weight markers in kD. The results shown are representative of three separate experiments.

clue came from two complementary approaches comparing the binding of PP14 to either ASF or to its sialylated form fetuin, using solid-phase binding and competition assays. These assays demonstrated opposite preferences of galectin-1 and PP14 in relation to the sialylation state. Fetuin is a glycoprotein carrying both α2,3- and α2,6-sialylated LacNAc sequences (Nilsson *et al.*, 1979). Galectin-1 preferentially binds to LacNAc sequences and can tolerate the presence of α2,3-linked sialic acid (Ahmad *et al.*, 2002; Leppanen *et al.*, 2005). However, the addition of α2,6-linked sialic acid abrogates its binding (Ahmad *et al.*, 2002; Amano *et al.*, 2003; Leppanen *et al.*, 2005). In structural detail, the conformation of bound sialylgalactose maintains a low-energy position (Siebert *et al.*, 2003). Hence, as expected, galectin-1 prefers to bind to the desialylated form of fetuin, ASF with fractional high affinities (Dam *et al.*, 2005). On the contrary, the data indicated a preferential binding of PP14 to fetuin, suggesting higher binding of PP14 to sialylated over desialylated epitopes. To more specifically test this first clue we have used distinct carbohydrates to compete PP14's binding to T cells. These experiments provided further support to PP14's selective binding to LacNAc sequences that is increased upon addition of α2,6-linked sialic acid. This result was further strengthened by the higher extent of binding of PP14 to neoglycoconjugates containing α2,6-linked sialylgalactose as compared with α2,3-sialylated isomer. Finally, sialidase treatment of CD4^{+} T cells resulted in reduced binding of PP14 to their surface and in parallel rendered these cells less sensitive to PP14-mediated inhibition. Hence, while the addition of α2,6-linked sialic acid to *N*-glycan branch ends on T-cell glycoproteins inhibits galectin-1 binding and function (Amano *et al.*, 2003), this modification is expected to enhance binding of PP14 to the cells and increase susceptibility of these cells to PP14-mediated inhibition.

The glycosylation state of T-cell surface glycoproteins is known to be regulated during T-cell maturation and activation (Whiteheart *et al.*, 1990; Krishna and Varki, 1997; Bagriacik and Miller, 1999; Priatel *et al.*, 2000; Daniels *et al.*, 2001) and reviewed in Lowe, 2001. In particular, alterations in the pattern of CD45 isoform expression that goes along with changes in glycosylation and sialylation accompany different T-cell activation states (reviewed in Poppema *et al.*, 1996). The present findings on PP14's lectin-like property, and especially the newly discovered preference of PP14 to Galβ1,4GlcNAc sequences with α2,6-linked sialic acid, enabled us to determine whether PP14's carbohydrate-binding profile correlates with binding to—and inhibition of—T cells of different subsets and activation states that are known to differ in their surface glycan displays. In this study, we have compared naïve versus activated/memory CD4^{+} T cells, sorted based on their CD45RA and CD45RO expression, respectively. Our data established that while PP14 inhibits the activation of both T-cell subsets, CD45RA T cells are significantly more sensitive to PP14-mediated inhibition as compared with CD45RO T cells.

Despite the significant difference in the functional effect of PP14 on the two T-cell subsets, PP14 bound both cell subsets equally well. One possible explanation for that is that PP14's binding to T-cell surfaces is carbohydrate-dependent and is not restricted to only one type of glycoprotein. However, its interaction with CD45 appeared to have functional significance (Rachmilewitz *et al.*, 2003). Thus, the higher sensitivity of naïve cells to PP14-mediated inhibition may depend on the specific CD45 glycoform expressed on these cells and PP14's preferential binding to SAα2,6Gal sequences and is not dependent on the overall binding to other surface glycoproteins. Indeed, it was suggested that CD45 is the preferred substrate for the α2,6-sialyltransferase (STGal-I), the enzyme that adds sialic acid in α2,6-linkage to Gal sequences (Amano *et al.*, 2003). Furthermore, in mature human thymocytes the SAα2,6Gal sequence was only detected on the CD45RA isoform (Baum *et al.*, 1996). Thus, given that PP14 preferentially binds to α2,6-sialylated LacNAc (in contrast to the galectin) PP14 may favor the naïve cells expressing the sialylated CD45RA glycoform.

To test this hypothesis, we specifically looked at PP14 association with CD45RA versus CD45RO isoforms on naïve and memory/activated cells, respectively. Using co-capping experiments, we established that PP14 preferentially associates with CD45RA, correlating with higher sensitivity of CD45RA^{+} T cells to PP14-mediated inhibition as compared with CD45RO^{+} T cells. Interestingly, galectin-1 with its restricted binding to LacNAc and its α2,3-sialylated derivative targets activated/memory T cells by binding to CD45RO (Baum *et al.*, 1995).

Previous study has demonstrated homodimerization of CD45 receptor in T cells and suggested that this process is

negatively modulated by sialylation (Rachmilewitz *et al.*, 2003). Hence, the least sialylated CD45RO isoform homodimerizes efficiently, while the highly sialylated isoform, CD45RA, dimerizes upon removal of sialic acid (Xu and Weiss, 2002). It has been suggested that this homodimerization of CD45 occurs spontaneously. Alternatively, binding of an endogenously expressed galectin to CD45 on the cell surface may induce its dimerization. According to this proposal sialylation, such as that decorating CD45RA, will mask a galectin's docking site and prevent or limit dimerization of this isoform. The scenario whereby an endogenous galectin induces receptor clustering has been previously demonstrated in the case of galectin-3 binding to TCR that restricts receptor aggregation and recruitment to the site of antigen presentation (Demetriou *et al.*, 2001). However, two salient differences between galectin-3 and the homodimeric galectin-1 deserve attention: this chimera-type galectin tolerates terminal $\alpha 2,6$-sialylation in LacNAc repeats, and the topology of its glycoconjugate cross-linking will not equal that of the proto-type galectins (Kopitz *et al.*, 2001; Ahmad *et al.*, 2002).

In our study the mobility shift of cross-linked CD45 did not appear as a single discrete band but rather as a broad band, suggesting that interactions between CD45 and other surface glycoproteins may take place in addition to homodimerization. In fact, galectins are known to bind several glycoproteins (such as CD2, CD3, CD7, CD43 and CD45 in the case of galectin-1) and were suggested to form galectin : glycoprotein lattices, a process that is being explored (Pace *et al.*, 1999; Sacchettini *et al.*, 2001; Villalobo *et al.*, in press). Our data demonstrated that exogenously added PP14 with its preferential binding to $\alpha 2,6$-sialylated LacNAc may similarly promote CD45 clustering/dimerization, in this case including also the sialylated glycoform CD45RA.

Taken together, these findings support two alternative models for PP14 function based on its lectin-like potential. The first straightforward model is based on regulation of the phosphatase activity by PP14 : CD45 interaction that promotes CD45 dimerization, with CD45RA isoform being a major target given PP14's preferential binding to $\alpha 2,6$-sialylated LacNAc. However, our data support other means of regulation of TCR signaling that are based on cellular localization of CD45 rather than regulation of its phosphatase activity. We have demonstrated that despite the importance of PP14 : CD45 interaction for PP14 activity, PP14's binding to the T cell is not limited to CD45 alone (Rachmilewitz *et al.*, 2003). Furthermore, we have previously demonstrated that PP14 translocates to APC : T-cell contact sites, where it exerts its inhibitory activities (Rachmilewitz *et al.*, 2002). Therefore, PP14 may form glycoprotein lattices at APC : T-cell contact sites. They in turn interfere with the two-dimensional organization of the immune synapse that usually includes exclusion of CD45 from the central region of the immune synapse and from membrane lipid rafts (Shaw and Dustin, 1997; Thomas, 1999; Van Laethem and Leo, 2002). This results in segregation of CD45 from its substrates leading to increased stability of TCR-induced phosphorylation in target proteins. In contrast, in the presence of PP14, PP14-glycoprotein

lattices may be formed at APC : T-cell contact sites, which in turn may interfere with the two-dimensional organization of the immune synapse and receptor clustering; the main consequence is the disruption of CD45 sequestration in the "immune synapse," leading to excess dephosphorylation of TCR-induced phosphoproteins (Rachmilewitz *et al.*, 2002) and elevated activation threshold of TCR signaling (Rachmilewitz *et al.*, 2001). According to this model, PP14-mediated regulation of TCR signaling is dependent on changing the local equilibrium between kinase and phosphatase activity within the contact site without changing overall CD45 phosphatase activity.

Materials and methods

Cells

PBMC were purified from the venous blood of healthy donors by density gradient centrifugation, as described (Rachmilewitz *et al.*, 1999). CD4$^+$ T cells were isolated by negative selection using the RosetteSep human CD4$^+$ T-cell enrichment cocktail (StemCell Technologies, Vancouver, Canada). CD45RA and CD45RO cells were further separated from the CD4$^+$ T cells using anti-CD45RA and anti-CD45RO microbeads, respectively, with magnetic cell isolation system (Miltenyi Biotec, Bergisch-Gladbach, Germany). All the experiments were performed with CD45RA and CD45RO T cells isolated by both negative and positive selection methods achieving similar results. CD45RA and CD45RO expression was measured by direct immunofluorescence using phycoerythrin (PE)-conjugated anti-CD45RA and FITC-conjugated anti-CD45RO mAb (IQ-Products, Groningen, The Netherlands), and the immunostained cells (2×10^4 cells/sample) were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using Cell Quest software. All the experiments were performed with CD45RA and RO T cells isolated by both negative and positive selection with similar results. As a source of APC, monocytes were isolated from peripheral blood mononuclear cell populations by the RosetteSep human CD14$^+$ enrichment cocktail (StemCell Technologies). The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Biological Industries, Beit-Haemek, Israel) supplemented with 10% heat-inactivated fetal calf serum (Biological Industries), 2 mM glutamine, and penicillin/streptomycin.

AF samples and production of PP14·Fc$_{\gamma 1}$

Discarded human AF samples were obtained from the Center for Human Genetics Laboratory at Hadassah Hospital and stored at –80°C. Samples obtained from several patients (collected at 16–18 weeks of gestation) were pooled and filter-sterilized before use. PP14·Fc$_{\gamma 1}$ was prepared as described (Rachmilewitz *et al.*, 2003), and each fraction preparation was tested for activity before subsequent use in experiments.

Preparation of galectin-1 and SAP and lectin labeling

Human galectin-1 was purified after recombinant production using affinity chromatography on lactosylated Sepharose 4B, obtained by divinyl sulfone activation, as

crucial step, and quality controls were performed by 1D and 2D gel electrophoresis, gel filtration, haemagglutination, and electrospray ionization mass spectrometry (He *et al.*, 2003; Kopitz *et al.*, 2003; Lopez-Lucendo *et al.*, 2004). Labeling was performed under activity-preserving conditions with biotinyl-*N*-hydroxysuccinimide ester, biotin incorporation was quantitatively determined by a proteomics protocol and maintenance of activity was ascertained by solid-phase assays (Andre *et al.*, 2001; Purkrabkova *et al.*, 2003). SAP was purified from human serum using affinity chromatography on mannosylated Sepharose 4B as crucial step, its lectin reactivity was routinely assayed to ascertain integrity of the single complex-type biantennary *N*-glycan attached to Asn32 of each protomer and biotinylation followed the protocol for lectin labeling (Andre *et al.*, 2001).

Detection of glycan-dependent binding of lectins to glycoproteins using solid-phase assay

Binding assays of PP14, galectin-1, and plant lectins were performed on 384 black MaxiSorp microtiter plates (Nunc, Rochester, NY). Fetuin and ASF (Sigma, St. Louis, MO) were dissolved to 100 µg/mL in phosphate-buffered saline (PBS), dispensed at 25 µL/well and incubated overnight at 4°C. On the following day, residual protein-binding sites were blocked with HEPES buffered saline/bovine serum albumin (HBS/BSA) (1%) for 1 h at room temperature, and the plate was then washed with HBS (Hepes 50 mM, pH 7; NaCl, 150 mM). The wells were next reacted with various protein samples, dispensed in quadruplicate at 25 µL/well. Biotinylated plant lectins were diluted to 5 µg/mL in HBS (SNA), ECA—Vector Laboratories, Burlingame, CA; concanavalin A (ConA; Sigma). PP14·Fc$_{\gamma l}$ was diluted in HBS/BSA (1%) with or without 10 mM CaCl$_2$ at a final concentration of 80 µg/mL and with 2.5 µg/mL biotinylated goat anti-human IgG Fc$_\gamma$ (Jackson ImmunoResearch, West Grove, PA). Galectin-1 was prepared in HBS/BSA (1%) at a final concentration of 20 µg/mL. Following a 1-h incubation at 37°C, the plate was washed with 10 mM HBS/CaCl$_2$, and 25 µL/well of 500 ng/mL DELFIA streptavidin-conjugated Europium (PerkinElmer, Turku, Finland) in HBS/CaCl$_2$ was then added and incubated at 37°C for 30 min. After rinsing with HBS/CaCl$_2$, 25 µL/well of DELFIA Enhancement Solution was added (PerkinElmer), and after 20-min incubation the plate was read in time-resolved fluorescence settings with Victor 1420 equipment (PerkinElmer; ex/em 340/612).

In parallel experiments, PP14 was the component immobilized to the surface of wells. In detail, the surface of Maxisorb microtiter plates (Nunc) was coated with PP14·Fc$_{\gamma l}$ (20 mg/mL in 20 mM Hepes pH 7.2) during incubation overnight at 4°C. After coating, each well was washed three times and to saturate residual protein-binding sites the wells were incubated with 20 mM Hepes pH 7.2 containing 1% BSA for an hour at 37°C. Biotin-labeled glycoproteins or biotin-conjugated polyacrylamide-based neoglycoconjugates (PAA; synthesome) were then added to the wells and incubated for an hour at 37°C. Following a washing step the wells were incubated with HRP-conjugated avidin (0.5 mg/mL) for an hour at 37°C and then washed and incubated with *o*-phenylenediamine/H$_2$O$_2$ substrate (Sigma). The reactions were stopped by adding 50 mL of 0.1 M H$_2$SO$_4$, and the optical density at 490 nm was measured. All experiments were done in triplicate, and the data are mean values of the results. The standard deviation did not exceed 10% and in most experiments was <5% of the mean value.

PP14·Fc$_{\gamma l}$ and galectin-1 binding to T cells

T cells (CD4$^+$, CD45RO, or CD45RA) were incubated with 2 µg/mL of either PP14·Fc$_{\gamma l}$ or CTLA-4·Fc$_{\gamma l}$ (R & D Systems, Minneapolis, MN) or 0.55 µg/mL of biotinylated galectin-1 for 30 min at 37°C, and the cells were then methanol fixed. After rehydration, probe binding to the fixed cells was determined with PE-conjugated F(ab)$_2$ fragment goat anti-human IgG (for Fc$_\gamma$ detection; Jackson ImmunoResearch) or FITC-conjugated streptavidin (for galectin-1 detection). About 1×10^4 cells/sample were analyzed on a FACS Calibure flow cytometer (Becton Dickinson) using Cell Quest software.

Sialidase treatment

CD4$^+$ T cells (1×10^7 cells/mL in PBS) were incubated at 37°C with or without 100 U/mL sialidase (*Vibrio cholerae*; Sigma). Following 30-min incubation, the cells were washed with RPMI. The efficiency of sialidase treatment was determined by direct immunofluorescence using FITC-conjugated SNA (Vector Laboratories), and the stained cells (2×10^4 cells/sample) were analyzed on a FACS Calibure flow cytometer (Becton Dickinson) using Cell Quest software.

Cytokine production and cell proliferation

Either 5×10^4 CD4$^+$ T cells or separated CD45RA and CD45RO subsets and 5×10^4 monocytes were cultured in flat-bottom 96-well plates (Corning, Corning, NY) in 0.2 mL volume per well in triplicate. Cells were stimulated with the superantigen SEB (1 ng/mL; Sigma) for 72 h in the presence or absence of either AF or PP14·Fc$_{\gamma l}$. Conditioned media were collected and IFN-γ levels were assayed by ELISA (R & D Systems). For cell proliferation assays, cultures were pulsed with [^{3}H]-methyl-thymidine (Amersham-Pharmacia Biotech, Buckinghamshire, England) that was added for the last 18 h. Cells were harvested onto glass-fiber filter paper (Schleicher & Schull, Dassel, Germany), the filters were dried, and the incorporated ^{3}H analyzed with liquid scintillation counter (Wallac, Gaithersburg, MD). Data points are expressed as mean of triplicate samples.

Fluorescence co-capping

Colocalization of PP14 and CD45 was assessed in co-capping experiments. Separated populations of CD45RA or CD45RO CD4$^+$ T cells were incubated with 2 µg/mL of either PP14·Fc$_{\gamma l}$ or CTLA-4 Fc$_{\gamma l}$ (R & D Systems) in the presence or absence of 10 µg/mL F(ab)$_2$ fragment goat anti-human IgG (Jackson ImmunoResearch). The cells were then fixed with methanol, and following rehydration, CD45 was detected with pan-specific anti-CD45 mAb (GAP8.3; ATCC) and Alexa-488-conjugated F(ab)$_2$ fragment goat anti-mouse IgG (Molecular Probes, Eugene, OR); PP14·Fc$_{\gamma l}$ was detected using Cy5-conjugated goat anti-human IgG (Jackson ImmunoResearch). Control cells (stained with

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Alexa-488- or Cy5-conjugated Ab alone) were always processed simultaneously for each experiment. Images were obtained using an laser scanning microscope confocal laser scanning system connected to a Zeiss Axiovert 135 M inverted microscope with a 100/1.3 plan-Neofluor lens. Cells were scanned by dual excitation of Alexa-488 (green) and Cy5 (red) fluorescence, with overlap of green and red fluorescence detected as a yellow signal. The frequency of areas of PP14 : CD45 colocalization and their size were calculated using Image Pro 4.5 (Media Cybernetics, Silver Spring, MD).

Cell surface cross-linking, cell lysis, and immunoblotting

CD45RA or CD45RO CD4$^+$ T cells (10×10^6/mL) were incubated with either AF (10% v/v) or PP14·Fc$_{\gamma l}$ (30 mg/mL) for 30 min at 37°C and were then transferred to ice for surface cross-linking using freshly prepared sulfo-EGS (Pierce, Rockford, IL) as previously described (Xu and Weiss, 2002). The cells were lysed as previously described (Rachmilewitz *et al.*, 2002), and whole cell lysates were analyzed by electrophoresis on 3–8% gradient gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The blots were probed with anti-CD45 mAb (GAP8.3; ATCC), processed with ECL plus western blotting detection system (Amersham-Pharmacia Biotech), exposed to Chemiluminescence BioMax Light Film (Kodak-Industries, Chalon-sur-Saône Cedex, France).

Abbreviations

AF, amniotic fluid; APC, antigen-presenting cells; ASF, asialofetuin; BSA, bovine serum albumin; ConA, concanavalin A; ECA, *Erytherina cristagalli* agglutinin; HBS, HEPES buffered saline; IFN-γ, interferon-γ; LacNAc, *N*-acetyllactosamine; PAA, polyacrylamide-based backbone; PE, phycoerythrin; PP14, placental protein 14; SAP, serum amyloid P component; SEB, staphylococcal enterotoxin B; SNA, *Sambucus nigra* agglutinin.

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