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Differential Regulation of Th1/Th2 Cytokine Responses by Placental Protein 14

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The potency of TCR signaling during primary CD4+ T cell activation influences initial cytokine expression patterns and subsequent polarization toward either Th1 or Th2 subsets. In this study, we demonstrate that the T cell inhibitor placental protein 14 (PP14; glycodelin) preferentially inhibits Th1 cytokine responses and chemokine expression when present during ex vivo priming of CD4+ T cells. PP14 synergizes with exogenously added IL-4 in skewing T cell responses. Significantly, PP14 impairs the down-regulation of GATA-3 transcriptional regulator expression that normally accompanies T cell activation, which is a prerequisite for Th1 development. Taken together, these data document for the first time the ability of PP14 to skew Th1 responses. The Journal of Immunology, 2004, 173: 5524-5530.

Naive CD4+ T cells can differentiate into distinct effector cell subsets, characterized by the production of cytokines that promote different types of immune responses. Th1 cells express the hallmark cytokines IL-2 and IFN-γ, which are linked to cell-mediated immunity, whereas Th2 cells predominantly express IL-4 and IL-5 and promote humoral immunity. The selective differentiation of either subset is established during priming and can be significantly influenced by a variety of factors. One of these factors, the cytokine environment, has been presented as the major factor influencing T help development. Hence, during in vitro activation, polarization of naive T cells can be driven to the Th1 or Th2 phenotype by the addition of IL-12 or IL-4, respectively, in conjunction with blocking Abs to the cytokine that drives the alternate phenotype. In addition, the potency of TCR signaling that depends on priming conditions such as the nature (i.e., agonist or altered peptide ligand) and dose of Ag as well as the presence of costimulation also contributes to Th1 vs Th2 phenotype choice (1). The costimulatory receptor, CD28, by triggering an active accumulation of various cell surface molecules to the interface of the T cell and the APC, contributes to signal strength and increases the amplitude and duration of TCR signaling (2, 3).

In contrast to the amplifying effects of costimulation, we have previously demonstrated that the immunomodulatory glycoprotein, placental protein 14 (PP14;3 also known as glycodelin (4)), translocates to APC:T cell contact sites, where it decreases stability of TCR-induced phosphoproteins (5). This latter activity of PP14 fits in well with the antagonism between CD28-mediated costimulation and PP14-mediated inhibition (6) as well as with recent data indicating that the inhibitory activity of PP14 is mediated by the tyrosine phosphatase receptor, CD45 (7). These findings help to explain the capacity of PP14 to desensitize TCR signaling and to operate via a unique immunoregulatory mechanism, rheostatically elevating TCR activation thresholds (6).

PP14 has other described activities outside the immune system, including the ability to inhibit sperm- zona interaction (8, 9), and to promote angiogenesis, possibly by promoting vascular endothelial growth factor expression (10). However, the immunomodulatory functions attributed to PP14 are of special interest in light of its tissue distribution. PP14 is expressed in maternal reproductive tracts and is highly abundant during early pregnancy, constituting up to 10% of total decidual protein in the first trimester, and occurs in remarkably high concentrations in amniotic fluid (AF) during the first half of pregnancy (125 mg/L; of decidual origin) (11, 12). Significant concentrations of PP14 are also present in tumors of reproductive organs (13, 14), in plasma during early pregnancy (12), and in male seminal fluid (15). Interestingly, decidual NK cells were shown to express PP14 (16), and in turn PP14 has been shown to suppress NK function (17). PP14 has also been identified outside of the reproductive system, within cells of the megakaryocytic lineage and platelets (18). Taken together, these findings may explain, at least in part, the correlation between PP14 serum levels and the establishment and progression of a normal pregnancy (19–21), and it is thus tempting to speculate that PP14 may contribute to maternal immunotolerance to the semiallogeneic embryo.

Based on our previous studies demonstrating the ability of PP14 to desensitize TCR signaling, we have now looked for differential effects by PP14 on Th1 vs Th2 responses during T cell priming. Our data demonstrated that whereas both Th1 and Th2 responses were inhibited by PP14, Th1 responses were overall more sensitive to inhibition. Mechanistically, PP14-treated cells showed impaired TCR-induced repression of the Th2-linked transcription factor, GATA-3. The data suggest that PP14 may preferentially antagonize Th1 commitment, hence skewing the response toward the Th2 phenotype.

Materials and Methods

Cells

Cells were purified from the venous blood of healthy donors. CD4+ T cells were isolated either by positive selection using anti-CD4 microbeads with
magnetic cell isolation system (Miltenyi Biotec, Bergisch Gladbach, Germany), or by negative selection using RosetteSep human CD4+ T cell enrichment mixture (StemCell Technologies, Vancouver, Canada). All of the experiments were performed with CD4+ T cells isolated by one of the two methods, achieving similar results. As a source of APC, monocytes were isolated from PBMC populations by either adherence to plastic, as previously described (6), or RosetteSep human CD4+ enrichment mixture (StemCell Technologies). The cells were maintained in RPMI 1640 medium (Biological Industries, Beit-Haemek, Israel) supplemented with 10% heat-inactivated FCS (Biological Industries), 2 mM glutamine, and penicillin/streptomycin.

Monocytes were washed with medium and resuspended at 10 × 10^6 cells/ml before being loaded with the superantigen staphylococcal enterotoxin B (SEB) at the indicated concentrations for 2 h at 4°C. The SEB-loaded cells were then washed three times with medium and diluted to 1 × 10^6 cells/ml in medium before adding them to culture.

**AF samples, PP14 immunoabsorption, and production of PP14•Fcyl**

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 wk of gestation) were pooled and filtered sterilized before use. Anti-PP14 polyclonal Ab (18) were obtained from several patients (collected at 16–18 wk of gestation) were discarded (22). PP14•Fcyl was prepared, as described (7), and each fraction preparation was tested for activity before subsequent use in experiments.

**Cytokine production**

 Cultures containing 10^6 CD4+ T cells and 10^6 monocytes loaded with various concentrations of the superantigen SEB, in the absence or presence of either AF or PP14•Fcyl, were plated in 1 ml of RPMI 1640 containing 10% FCS in individual wells of a 24-well culture plate. Cells were stimulated for the indicated times, and conditioned medium was collected. IL-2, IL-4, IL-5, IL-13, and IFN-γ were also evaluated at a series of time points (between 5 h and 10 days), but their levels proved to be below the sensitivity of the ELISA (31.25 and 62.5 pg/ml, respectively; data not shown).

**Stimulation with SEB**, an agent that classically polarizes T cell responses toward the Th1 phenotype (23–25), actually promotes the secretion of a mixture of Th1/Th2 cytokines early on. This is reflected in the observed dose-dependent increases in secreted IFN-γ (Fig. 1A, upper panel), IL-2 (data not shown), and IL-5 (Fig. 1A, lower panel). However, the patterns for the Th1 vs Th2 cytokine responses differed. In the case of the Th1 cytokine IFN-γ, there was only minimal secretion at the lowest dose of SEB (1 ng/ml), and the dose response to higher concentrations of SEB was steep. In contrast, the Th2 cytokine IL-5 was inhibited by AF in some experiments (Fig. 1, A and B). Fig. 1B presents data from six independent experiments, demonstrating the consistency of the differential AF effects on IFN-γ vs IL-5 responses, with greater sensitivity of IFN-γ to AF-mediated inhibition, notwithstanding interexperiment variability (with different cell and AF donors). Of note, the AF used in this study did not contain detectable levels of the cytokines under study (data not shown).

Importantly, we have previously demonstrated that PP14 accounts for the T cell inhibitory activity AF (22). In line with this report, AF that had been immunodepleted of its PP14 did not inhibit IFN-γ or IL-5 secretion (Fig. 1C), suggesting that PP14 is indeed the active factor within AF. This is consistent with our previous finding that AF depletion of its PP14 no longer inhibits IL-2 or T cell proliferation (6). Furthermore, a rPP14 derivative, PP14•Fcyl, showed similar inhibitory effects (Fig. 1D). PP14•Fcyl significantly inhibited the secretion of IFN-γ at all SEB doses tested, while having only a marginal effect on IL-5 secretion. For these experiments, the data have been normalized against a control Fcyl fusion protein, CD99•Fcyl, which itself inhibited IL-5 secretion to some extent, especially at higher SEB doses, although it had only a marginal effect on IFN-γ secretion. The basis for this IL-5 inhibitory effect is unclear.

As a next step, we examined AF and PP14•Fcyl effects on levels of intracellular cytokines (IFN-γ and IL-4) to validate the cytokine secretion findings at the single cell level. SEB-loaded monocytes were used to stimulate CD4+ T cells, in this case for
only 24 h, as the optimal time point for detecting IL-4-expressing cells. Cells were treated with monensin for the last 5 h of incubation, and then fixed, permeabilized, and double immunostained with cytokine-specific Abs. Given that only ∼10% of T cells express the TCR Vβ subtype that mediates SEB responsiveness, a relatively low percentage of immunodetectable, cytokine-expressing cells is expected. As shown in Fig. 1E, SEB induced a dramatic increase in the percentage of cells with intracellular IFN-γ. The increase in the percentage of IL-4-expressing cells was less marked, and in fact, in some experiments, IL-4 cells actually decreased at the highest SEB concentration (data not shown). Importantly, both AF and PP14•Fcγ1 significantly decreased the percentage of IFN-γ+ cells, without substantially changing the low levels of IL-4+ cell in the population (Fig. 1E). Thus, intracellular cytokine findings paralleled those with secreted cytokines, and extended the findings to a second Th2 cytokine, IL-4.

The differentiation of Th cells is influenced by regulatory cytokines, such as IL-4 and IL-12, which are present within the cells’ microenvironment during the activation process. Because IL-4 is known to polarize Th cells toward the Th2 phenotype, we compared the effects of exogenous IL-4 vs PP14 on cytokine expression under these short-term, nonpolarizing experimental conditions. To this end, CD4+ T cells were stimulated as before with SEB-loaded monocytes, and cytokine secretion (Fig. 2A) and percentage of cells expressing intracellular cytokine (Fig. 2B) were assessed. As was the case with AF and PP14, addition of exogenous IL-4 decreased IFN-γ secretion, while moderately increasing IL-5 secretion (Fig. 2A). Significantly, there was a pronounced additive effect on IFN-γ secretion when AF...
or PP14•Fcγ1 was used in combination with exogenous IL-4 (Fig. 2A, left panel). In contrast, AF actually augmented the moderate increase in IL-5 levels induced by IL-4 in the presence of high SEB concentrations; thus, AF and IL-4 in combination promoted even greater IL-5 secretion (Fig. 2A, right panel). Of note, at lower SEB concentrations, in which PP14 inhibited IL-5 secretion, the addition of exogenous IL-4 overrode this PP14-mediated inhibition (data not shown).

Intracellular cytokine analysis yielded similar parallels between exogenous IL-4 and PP14. Addition of exogenous IL-4 or PP14•Fcγ1 (or AF) each reduced the percentage of IFN-γ cells, and notably, when used in combination, these two agents resulted in an even greater decrease in the percentage of IFN-γ cells (Fig. 2B). In contrast, the percentage of IL-4+ cells was not substantially impacted by the addition of PP14•Fcγ1 (or AF), with or without exogenous IL-4. Taken together, these findings demonstrate a parallel between PP14 and exogenous IL-4 on Th1 vs Th2 cytokine production and secretion under nonpolarizing conditions, along with a significant combinatorial effect when used together.

Chemokines, like cytokines, mold Th cell responses. As T cells differentiate along the Th1 and Th2 pathways, they acquire distinctive migratory capabilities, which are coupled to their chemokine receptor expression (30). We first surveyed the expression of a small panel of chemokine receptors (CXCR3, CCR5, CCR3, CCR7, and CXCR4) on CD4+ T cells following SEB stimulation. Only CXCR3 and CXCR4 demonstrated significant up-regulation in this experimental context. However, the expression of CXCR3 upon stimulation was limited to only subpopulation of the CD4+ T cells, corresponding to blast-transformed cells. In contrast, all CD4+ T cells express low levels of CXCR4, which increases upon SEB stimulation. Next, the effects of AF and PP14•Fcγ1 on the surface expression of these two chemokine receptors were determined, using SEB-stimulated and nonstimulated CD4+ T cells in side-by-side analyses. As shown in Fig. 3, both AF and PP14•Fcγ1 differentially affected the two chemokine receptors, reducing the number of cells expressing CXCR3, but not CXCR4. This chemokine receptor finding is consistent with the previous cytokine findings, in that PP14 preferentially inhibits the chemokine receptor expressed at high levels on Th1 cells and very low levels on Th2 cells (CXCR3), but not CXCR4. This chemokine receptor finding is consistent with the previous cytokine findings, in that PP14 preferentially inhibits the chemokine receptor expressed at high levels on Th1 cells and very low levels on Th2 cells (CXCR3), but not the chemokine receptor expressed at varying levels on Th2 cells, but not at all on Th1 cells (CXCR4) (31, 32).

To further evaluate the influence of PP14 on Th differentiation, we determined its effect on expression of the transcription factor GATA-3, a major regulator of Th2 differentiation. GATA-3 expression is regulated by TCR-induced signals (33, 34). It is expressed at high levels in naive T cells and Th2 lineage cells, but is down-regulated when stimulated under conditions that promote Th1 differentiation. Furthermore, preventing GATA-3 down-regulation after activation maintains the Th2 phenotype and prevents Th1 differentiation (35). This suggests that the repression of GATA-3 is required for progression toward a Th1 phenotype (36).

To examine the effects of PP14 on TCR-induced GATA-3 expression, we stimulated CD4+ T cells under nonpolarizing (no added cytokines) conditions, in the presence or absence of PP14•Fcγ1 (or AF), and GATA-3 mRNA expression was determined by semi-quantitative RT-PCR analysis. Significantly, the down-regulation of GATA-3 after SEB stimulation was abrogated by the presence
PP14 selectively inhibits the expression of CXCR3, but not CXCR4 (Fig. 3A). Furthermore, these GATA-3 mRNA findings were corroborated at the protein level by immunofluorescence and flow cytometric analysis of intracellular GATA-3 (Fig. 4B). Thus, while GATA-3 protein was reduced after SEB stimulation, PP14•Fcγ1 prevented this down-regulation, maintaining it at the level found in unstimulated cells. This PP14-mediated interference with the repression of a transcription factor required for Th1 differentiation is consistent with a role for PP14 in promoting a Th2 phenotype.

Taken together, these results support a model wherein PP14 differentially inhibits Th1 vs Th2 responses during T cell priming, hence driving the polarization of Th2 cells.

**Discussion**

PP14 (glycodelin) is a pregnancy-associated protein with unique immunoregulatory properties. Our early data established that PP14 acts directly on T cells, targeting early events during TCR signaling (22). PP14 decreases the stability of TCR-induced phosphoproteins (5), which is linked to desensitization of TCR signaling and elevation of T cell activation thresholds (6). In this way, PP14 has opposite effects to costimulation through CD80 (37). Other mechanistic clues lie in the dependence of the T cell inhibitory activity of PP14 on the surface phosphatase CD45 (7), suggesting that PP14 may somehow interfere with the transit of CD45 and perhaps other components in and out of APC:T cell contact sites (5).

Knowing that PP14 desensitizes TCR signaling (6), and that the strength of TCR signaling dictates how T cell responses evolve (1, 26–29), we now hypothesized that PP14 may differentially affect Th1 vs Th2 commitment during primary T cell activation.

The specific findings bearing upon the impact of PP14 on Th1 vs Th2 responses include the following: 1) Th1 (IFN-γ) and Th2 (IL-5 and IL-4) cytokine responses, as determined by cytokine secretion and intracellular cytokines, exhibit differential sensitivity to PP14-mediated inhibition; 2) PP14 parallels exogenous IL-4, a well-established Th2-polarizing cytokine, in its ability to skew cytokine responses toward a Th2 phenotype under nonpolarizing conditions; 3) PP14 selectively inhibits the expression of a chemokine receptor (CXCR3) associated with cells of the Th1 subtype, but not one of the Th2 subtype (CXCR4); 4) PP14 prevents the repression of the transcriptional factor GATA-3, an event that is essential for differentiation along the Th1 lineage.

The early balance between Th1 and Th2 cytokine output during primary T cell activation is dictated in part by the strength of TCR signaling, which in turn is influenced by factors such as antigenic dose and the nature of the Ag (1, 26–29). Thus, while both Th1 and Th2 cytokines are expressed simultaneously, low doses of stimuli primarily induced Th2 cytokines (IL-4 and IL-5), with low to undetectable levels of Th1 cytokines (IL-2 and IFN-γ), whereas priming with higher doses of stimuli favored increased production of Th1 cytokines. The Th1 skewing was correlated with the induction of ERK phosphorylation (26), and blocking this phosphorylation event specifically blocks the Th1 response (38). The observed effects of PP14 on early cytokine output can be readily understood within this framework. As one would have predicted for an agent that can attenuate the strength of TCR signaling, PP14, at each antigenic dose, tips the balance in favor of Th2 cytokines that require less TCR triggering. Accordingly, the low level IFN-γ production elicited by intermediate doses of SEB is completely ablated by PP14, in contrast to the IL-4 and IL-5 production at these doses of SEB, which are only partially blocked by PP14. At high doses of SEB, IFN-γ secretion is significantly inhibited by PP14, while IL-4 and IL-5 production is only slightly affected or even augmented.

This study focused on early events after T cell encounter with Ags, and thus, the experiments were performed under nonpolarizing conditions and with a relatively short duration of Ag exposure.
Nonetheless, our findings in this context are most likely relevant to polarization. Due to the cross-regulatory and self-propagating properties of cytokines, IL-4 and IFN-γ in particular (1), the early secretion of such cytokines, and their balance, soon after T cell stimulation, is a major determinant in directing polarized responses. Thus, even minor changes in the early cytokine microenvironment elicited by agents such as PP14 are likely to stochastically influence population dynamics and polarize responses over time skewed to the Th2 subset.

The cytokine secretion and intracellular cytokine data pointing to the contribution of PP14 to Th2 skewing were reinforced by analysis of the GATA-3 transcriptional factor, which plays a pivotal role in Th2 differentiation. Our key finding was that PP14 blocks TCR-induced down-regulation of GATA-3 expression in CD4+ T cells, which conveniently explains PP14’s preferential inhibition of Th1 responses and skewing toward Th2 cytokine output. Normally, GATA-3 expression is high in naive T cells and down-regulated upon TCR triggering. Importantly, GATA-3 is strongly expressed in Th2, but not Th1 cells. This expression profile is functionally significant, because GATA-3 directly inhibits Th1 cytokines by a cell-intrinsic mechanism and independently augments Th2-specific cytokines (39). A feedback pathway is established that stabilizes Th2 commitment and antagonizes Th1 commitment (40). GATA-3 has thus emerged as a master switch in inhibition of Th1 responses and skewing toward Th2 cytokine output. Interestingly, pregnancy is reportedly associated with a Th2 shift in humans, and this could be important in the protective effect of pregnancy, because cell-mediated autoimmunity is driven by Th1 responses. PP14, which is present at high levels in maternal serum, could thus be a critical driver in pregnancy’s Th2 shift and the associated improvement in autoimmunity. Another recent immunological observation is that the predominant NK lymphoid subset within the decidua of pregnancy expresses PP14 (16). This provocative finding, along with the high abundance of PP14 in AF, raises the possibility of additional contributions of PP14 to pregnancy and fetal protection.

### References


