

# Aberrant prostaglandin synthase 2 expression defines an antigen-presenting cell defect for insulin-dependent diabetes mellitus

S.A. Litherland,<sup>1</sup> X.T. Xie,<sup>1</sup> A.D. Hutson,<sup>2</sup> C. Wasserfall,<sup>1</sup> D.S. Whittaker,<sup>1</sup> J.-X. She,<sup>1</sup> A. Hofig,<sup>1</sup> M.A. Dennis,<sup>1</sup> K. Fuller,<sup>1</sup> R. Cook,<sup>1</sup> D. Schatz,<sup>3</sup> L.L. Moldawer,<sup>4</sup> and M.J. Clare-Salzler<sup>1</sup>

<sup>1</sup>Department of Immunology, Pathology, and Laboratory Medicine, College of Medicine,

<sup>2</sup>Department of Statistics, Division of Biostatistics,

<sup>3</sup>Department of Pediatrics, and

<sup>4</sup>Department of Surgery, College of Medicine, University of Florida, Gainesville, Florida 32610, USA

Address correspondence to: M.J. Clare-Salzler, Box 100275 JHMHC, Department of Pathology, College of Medicine, University of Florida, Gainesville, Florida 32610, USA. Phone: (352) 392-9885; Fax: (352) 846-2149; E-mail: salzler.pathology@mail.health.ufl.edu.

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Prostaglandins (PGs) are lipid molecules that profoundly affect cellular processes including inflammation and immune response. Pathways contributing to PG output are highly regulated in antigen-presenting cells such as macrophages and monocytes, which produce large quantities of these molecules upon activation. In this report, we demonstrate aberrant constitutive expression of the normally inducible cyclooxygenase PG synthase 2 (PGS<sub>2</sub>/COX-2) in nonactivated monocytes of humans with insulin-dependent diabetes mellitus (IDDM) and those with islet autoantibodies at increased risk of developing this disease. Constitutive PGS<sub>2</sub> appears to characterize a high risk for diabetes as it correlates with and predicts a low first-phase insulin response in autoantibody-positive subjects. Abnormal PGS<sub>2</sub> expression in at-risk subjects affected immune response in vitro, as the presence of a specific PGS<sub>2</sub> inhibitor, NS398, significantly increased IL-2 receptor  $\alpha$ -chain (CD25) expression on phytohemagglutinin-stimulated T cells. The effect of PGS<sub>2</sub> on CD25 expression was most profound in subjects expressing both *DR04* and *DQB0302* high-risk alleles, suggesting that this cyclooxygenase interacts with diabetes-associated MHC class II antigens to limit T-cell activation. These results indicate that constitutive PGS<sub>2</sub> expression in monocytes defines an antigen-presenting cell defect affecting immune response, and that this expression is a novel cell-associated risk marker for IDDM.

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## Introduction

Antigen-presenting cells (APCs) strongly influence several qualitative and quantitative aspects of T-cell activation (1–8). In humans at risk for insulin-dependent diabetes mellitus (IDDM), and in the nonobese diabetic (NOD) mouse, defects in APCs contribute to low levels of T-cell activation, poor IL-2 production, and deficient activation of regulatory T cells (9–13). Such APC defects may predispose to autoimmunity through quantitative reduction in signals required for activation-induced T-cell death (AICD) or regulatory T-cell responses, both of which are important mechanisms for peripheral tolerance (5, 14, 15).

Factors contributing to APC dysfunction in IDDM of humans, and in the NOD mouse, the murine model for this disease, include those encoded by the MHC class II region and non-MHC alleles. The unique H-2<sup>s7</sup> molecule of the NOD mouse plays a central role, as immunotolerogenic defects most readily occur in H-2<sup>s7</sup> homozygous NOD mice and IDDM rarely develops in congenic stocks of NOD heterozygous for other MHC haplotypes (16–18). In addition to the MHC, multiple unidentified non-MHC susceptibility genes contribute to the pathogenesis of IDDM in the NOD mouse and in humans

(19). The identities of these genes, and their contributions to lymphocyte and APC dysfunction, however, have not been defined.

Some studies suggest that heightened prostaglandin (PG) metabolism by macrophages may contribute to non-MHC-encoded APC dysfunction (20–22). PGs are lipid molecules derived from arachidonic acid; the rate-limiting step in their production is mediated by the cyclooxygenase PG synthase (PGS) (23, 24). There are 2 forms of this enzyme: PGS<sub>1</sub>, with constitutive expression in most cells, and PGS<sub>2</sub>, an inducible form found in a limited number of cell types such as macrophages and monocytes. PGS<sub>1</sub> is considered a homeobox gene necessary for homeostatic control of hormone responsiveness, whereas PGS<sub>2</sub> is an immediate-early gene activated in response to specific stimuli and with a tightly regulated pattern of expression (23–26).

Monocytes and macrophages do not express PGS<sub>2</sub>, and produce only low levels of PGs in the resting state. However, upon activation with agents such as LPS, these cells express PGS<sub>2</sub> and markedly increase PG output (24, 27, 28). Monocyte PGS<sub>2</sub> is expressed within 6 hours of activation and then shut off 16 hours after activation (29,

30). The proinflammatory PGs (e.g., PGE<sub>2</sub>), produced in abundance by macrophages and monocytes expressing PGS<sub>2</sub>, are potent modulators of the immune response and tolerance mechanisms (9, 31–37).

Recent work suggests that enhanced prostanoid metabolism in female NOD mice arises as a result of constitutive macrophage expression of PGS<sub>2</sub> (ref. 38; X.T. Xie, unpublished data). At first glance, enhanced prostanoid production in the NOD mouse would appear to be beneficial, as PGE<sub>2</sub> promotes Th2 responses *in vitro* (34, 35, 37) and suppresses IL-12 production (39), both of which are associated with protection from diabetes in the NOD mouse (40–42). However, reducing macrophage PGE<sub>2</sub> production *in vivo*, either by dietary fatty acid manipulation (22) or by treating NOD mice with indomethacin to block cyclooxygenase activity, significantly reduces diabetes incidence in female NOD mice by 70% and 50%, respectively (X.T. Xie, unpublished data).

The findings in the NOD mouse, suggesting a central role for PGS<sub>2</sub> in the pathogenesis of diabetes, prompted us to examine the expression of this enzyme in human monocytes. Similar to the NOD mouse, we found that constitutive PGS<sub>2</sub> expression was significantly greater in monocytes of subjects with IDDM, those at risk for the disease, and their relatives than in monocytes of healthy controls. Furthermore, monocyte PGS<sub>2</sub> expression correlated inversely with low insulin secretory reserve, suggesting that subjects expressing this enzyme are at high risk for IDDM. Aberrant PGS<sub>2</sub> expression severely limited *in vitro* T-cell activation, especially in individuals with IDDM-associated *DR04* and *DQβ0302* MHC class II alleles. These results are discussed with regard to the role of PGS<sub>2</sub> in the immunopathogenesis of IDDM and the prediction and prevention of this disease.

## Methods

**Materials.** Endotoxin-free Ficoll-Hypaque was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). PBS stock (1× solution; Sigma Chemical Co.) was made from endotoxin-free 10× solution (GIBCO BRL, Grand Island, New York, USA). RPMI-1640 (GIBCO BRL) plus glutamine was reconstituted in Milli-Q water (Millipore Corp., Bedford, Massachusetts, USA) and supplemented with 2 g/L sodium bicarbonate (Baker reagent grade; Fisher Scientific, Orlando, Florida, USA), 10% (vol/vol) heat-inactivated endotoxin-free FBS (HyClone Laboratories, Logan, Utah, USA), and 1% (vol/vol) penicillin, streptomycin, and neomycin (Sigma Chemical Co.), adjusted to pH 7.4. LPS (1–10 μg/mL) and phytohemagglutinin (PHA) (5–10 μg/mL) were purchased from Sigma Chemical Co. NS398, a specific PGS<sub>2</sub> inhibitor (43), was purchased from Cayman Chemical (Ann Arbor, Michigan, USA) and used at a 5-μM concentration. ELISA kits for detection of PGE<sub>2</sub> were purchased from Cayman Chemical. ELISA kits for IL-2 were purchased from Genzyme Pharmaceuticals (Cambridge, Massachusetts, USA).

**Antibodies.** FITC-conjugated mouse anti-human PGS<sub>2</sub> mAb (IgG1) was purchased from Cayman Chemical. Mouse mAb conjugates (phycoerythrin or FITC) against human CD14 (IgG2a/IgGb), CD69 (IgG1), pan-DR (Tu36;IgG2b), CD80 (IgG1), CD86 (IgG1), TNF-α

(IgG1), IL-10 (IgG2a), CD25 (IgG1), CD4 (IgG1), and CD8 (IgG1) were purchased from PharMingen (San Diego, California, USA) or Becton Dickinson Immunocytometry Systems (San Jose, California, USA). Anti-human CD105 FITC-labeled mAb (IgM) was a gift of M. Schnieder (University of Dusseldorf, Ulm, Germany). Human blood antigen-absorbed mouse isotype control antibodies were purchased from Sigma Chemical Co., Becton Dickinson Immunocytometry Systems, PharMingen, and Caltag Laboratories Inc. (Burlingame, California, USA). All antibodies were used at working concentrations of 1 μg/million cells.

**Human subject populations.** PBMCs were obtained from 70 subjects (ages 3–75 years; 37 female and 33 male) participating in the University of Florida Subcutaneous Insulin Diabetes Prevention Trial (SQ) and the Natural History of Diabetes Study (NH). Individuals in these trials were studied at 3- to 6-month intervals. Subjects were sampled twice on average for PGS<sub>2</sub> expression, with at least a 3-month interval between samplings. Subjects using PGS<sub>2</sub>-inhibitory drugs (e.g., nonsteroidal anti-inflammatory drugs or glucocorticoids), or with active inflammatory disease or infections at the time of sampling, were excluded from the studies. The SQ subjects received daily neutral protamine Hagedorn insulin injections (0.1–0.25 U/kg/d) that were discontinued 72 hours before evaluation. Almost all subjects included in the NH and SQ groups were relatives of IDDM patients, with varying degrees of risk for IDDM. Subjects were considered at high risk (HIGH) for IDDM if they were positive for islet cell autoantibodies (ICAs) or 2 or more autoantibodies (insulin autoantibodies [IAAs] or antiglutamate decarboxylase [GAD]) and had 1- and 3-minute insulin levels after intravenous glucose tolerance testing (IVGTT; first-phase insulin response [FPIR]) below the fifth percentile (<75 μIU/mL). Double autoantibody-positive or ICA<sup>+</sup> individuals with FPIR results above the threshold values were classified as moderate risk (MOD), and those with 1 autoantibody (e.g., IAA<sup>+</sup> or GAD<sup>+</sup> alone) were classified as low risk (LOW). ICA<sup>+</sup> subjects with clinically established diseases (Hashimoto's thyroiditis, Addison's disease, Graves' disease, vitiligo, ulcerative colitis, or rheumatoid arthritis) were also studied, and are designated autoimmune (AI). Oral glucose tolerance testing (OGTT) was performed at the time of each sampling to assess glucose intolerance and diabetes per National Diabetes Data Group criteria (44).

Ninety control samples were obtained from 24 healthy laboratory or clinic personnel (ages 18–55 years; 12 female and 12 male) who did not have a personal or family history of autoimmune diseases (IDDM, thyroid disease, vitiligo, Addison's disease, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, or inflammatory bowel disease). Samples were also obtained from 4 nondiabetic ICA<sup>-</sup> relatives of patients with established IDDM or other autoimmune diseases (ages 35–45 years; 1 female and 4 male). The investigators were blinded to family history of IDDM, *DR/DQβ* HLA alleles, IVGTT results, and autoantibody status.

**PBMC preparation and PGS<sub>2</sub> detection by flow cytometry.** A flow cytometric assay was developed for detection of intracellular PGS<sub>2</sub> in fixed and permeabilized PBMCs.

This method enabled detection of PGS<sub>2</sub> in subpopulations of PBMCs, e.g., CD14<sup>+</sup> monocytes (Figure 1) and concomitant analysis of monocyte markers and activation antigens. Furthermore, PBMCs were rapidly processed into azide-containing buffers (<90 minutes from the time of collection), reducing the potential for induction of PGS<sub>2</sub> protein, which occurs within 3–4 hours after monocyte activation (24, 27). With rare exception, blood samples from subjects and controls were obtained at the same time and analyzed in parallel.

Because PGS<sub>1</sub> and PGS<sub>2</sub> molecular homology is extensive, we used an established mAb recognizing a nonhomologous 18-amino acid sequence unique to PGS<sub>2</sub> (25, 26). Incubation of the PGS<sub>2</sub> mAb with the immunizing peptide, but not control peptide, completely blocked detection of PGS<sub>2</sub> by flow cytometry in activated monocytes.

PBMCs were isolated by centrifugation (500 g for 30 minutes at 25 °C) on Ficoll gradients, washed with 1× PBS, and resuspended in RPMI-1640 plus endotoxin-free 10% FCS. The PBMCs were counted, their viability was assessed by trypan blue exclusion, and they were diluted to 0.5 × 10<sup>6</sup> cells/200 μL in FACS buffer (PBS containing 1% [wt/vol] RIA-grade BSA and 0.1% [wt/vol] sodium azide, both from Sigma Chemical Co.). Positive controls for monocyte activation and PGS<sub>2</sub> expression were generated from aliquots of PBMCs cultured with 10 μg/mL LPS for 16–24 hours.

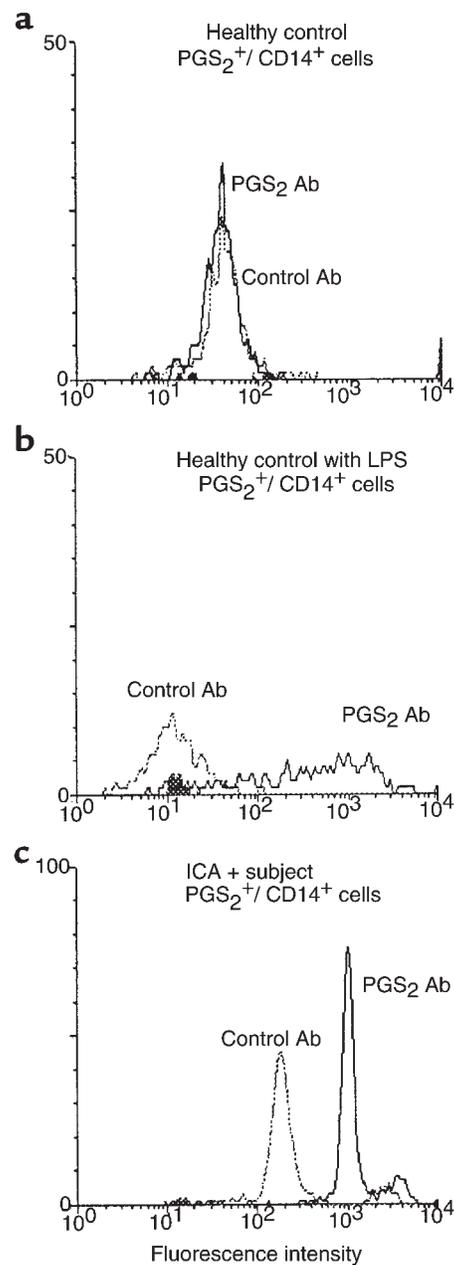
For intracellular detection of PGS<sub>2</sub> by flow cytometry, PBMCs were incubated in FACS buffer with endotoxin-free lyophilized mouse serum (Sigma Chemical Co.) reconstituted in endotoxin-free water (GIBCO BRL; 20 μg/million cells). This was supplemented with 10 μL of autologous human plasma per 100 μL of cell suspension. After 20 minutes, antibodies to surface antigens (e.g., CD14 or the appropriate isotype control antibody) were added to aliquots of cells and incubated for an additional 20 minutes. Cells were then fixed with 4% formaldehyde for 20 minutes. Fixed cells were washed and permeabilized with 0.5% (wt/vol) saponin (Sigma Chemical Co.) in FACS buffer. Anti-PGS<sub>2</sub>-FITC antibody or isotype antibody control was added, and cells were incubated for 1 hour at room temperature. All tubes were then washed 3 times with saponin buffer and finally suspended in FACS buffer. Flow cytometric analysis was performed using a Becton Dickinson FACSsort analyzer, collecting at least 10,000 ungated events. Cells positive for a given antigen were defined as those with a fluorescence intensity above that of cells stained with the corresponding isotype control antibody.

**Determination of monocyte activation.** Monocyte activation was determined from fresh PBMCs and from LPS-stimulated samples prepared in the manner just described. CD14<sup>+</sup> cells were analyzed for the concomitant expression of the following activation markers: CD69, CD105, DR, and intracellular TNF-α and IL-10.

**T-cell activation.** PBMCs were cultured in polypropylene tubes with medium alone or supplemented with 5 μg/mL PHA in the presence or absence of 5 μM of NS398. The cultures were maintained at 37 °C in 5% CO<sub>2</sub> for 16–24 hours before analysis. PBMCs were analyzed by flow cytometry for CD25 expression on CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells. Supernatants from cultures were ana-

lyzed for IL-2 and PGE<sub>2</sub> by ELISA. The effects of PG production on cytokine production and CD25 expression were determined by comparing cells cultured with PHA alone with those cultured with PHA and NS398.

**Statistical analysis.** A weighted Tobit analysis (45) was used to compare percentiles of controls, relatives, and cases across qualitative data that contained a significant mix of



**Figure 1**

Flow cytometric analysis of PGS<sub>2</sub> expression in CD14<sup>+</sup> monocytes from freshly isolated human PBMCs. Broken line indicates control isotype fluorescence; solid line indicates anti-PGS<sub>2</sub> fluorescence. (a) An example of PGS<sub>2</sub> expression in CD14<sup>+</sup> monocytes of freshly isolated PBMCs from a healthy control. (b) PGS<sub>2</sub> expression in LPS-activated (5 mg/mL for 24 hours) CD14<sup>+</sup> monocytes from the same individual as in a. (c) PGS<sub>2</sub> expression in CD14<sup>+</sup> monocytes of freshly isolated PBMCs from a subject at high risk for IDDM. Please note that the scales of events (y-axis) differ between the control and subject panels.

zero and nonzero percentage of PGS<sub>2</sub>/CD14<sup>+</sup> cell data. The Tobit analysis assumes that the nonzero data within each group approximates the uncensored portion above zero of a normal distribution. Because subjects in this study had varying numbers of measurements, ranging from a low of 1 measurement to a high of 20 measurements, a weighting scheme was used. The weighted scheme controlled for the contribution to the log likelihood for each observation; i.e., each subject's contribution to likelihood was equally weighted, as opposed to each observation being equally weighted. In addition, DQ, DR, IVGTT, and the covariates age and sex were also examined within the same model. A subgroup analysis of the cases was carried out using the same methodology.

Receiver operating characteristic (ROC) analysis (46) was used to examine the property of peak PGS<sub>2</sub> expression as a predictor of high risk for IDDM. A high risk of IDDM, as assessed by a low FPIR, was modeled using logistic regression as a function of peak PGS<sub>2</sub> expression. Covariate adjustments for sex, age, time between IVGTT and PGS<sub>2</sub> analyses, and genetic predisposition for IDDM (e.g., DR/DQB screening) were also included in the model. The ROC curve was then generated along with the corresponding c-statistic (area under the ROC curve). Kendall's  $\tau$ -b test was used for correlation analysis between PGS<sub>2</sub> expression and clinical parameters used in assessing clinical risk for diabetes. Data from the T-cell analyses were statistically analyzed using a Student's *t* test and ANOVA, as appropriate. When more than 1 sample from the T-cell analyses was available for an individual, these values were averaged and the mean used for comparison and statistical testing.

## Results

**PGS<sub>2</sub> expression in PBMCs.** Consistent with previous reports, the percentage of CD14<sup>+</sup> monocytes from healthy controls expressing PGS<sub>2</sub> was low in freshly isolated PBMCs (2.1 ± 3.9%; *n* = 24; Figure 2), but was markedly increased (Figure 1b) after 16–24 hours of LPS activation (56.4 ± 9.9%; *n* = 4). These findings were con-

firmed by fluorescent microscopy (data not shown).

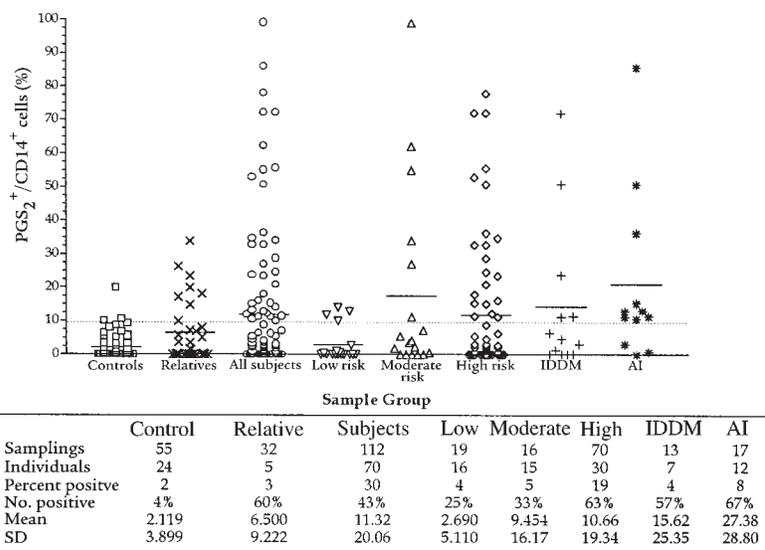
In marked contrast to control PBMCs, those from all subjects (individuals at risk for IDDM, patients with IDDM, and those designated AI (Figures 1c and 2) showed a significant increase in the percentage of CD14<sup>+</sup> monocytes expressing PGS<sub>2</sub> (*P* = 0.0032; Figure 2). Multivariate analysis of these data indicated that age and sex did not significantly influence the level of monocyte PGS<sub>2</sub> expression between groups.

Because the subject population examined contained subgroups with various levels of risk for IDDM, we compared PGS<sub>2</sub> expression among these groups. Using control population data, a cut point for high-level PGS<sub>2</sub> expression was established as the control mean plus 2 SDs (9.9% CD14<sup>+</sup>/PGS<sub>2</sub><sup>+</sup> cells). By this criteria, only 8% of controls (2/24) were positive for PGS<sub>2</sub>, whereas monocytes from 19 (63%) of 30 subjects at high risk for IDDM expressed this enzyme (Figure 2). Similar levels of monocyte PGS<sub>2</sub> expression were also found in established IDDM subjects (57%) and in ICA<sup>+</sup> individuals with other autoimmune diseases (67%).

Because IDDM subjects and those in the SQ prevention trial are treated with insulin, it is possible that this hormone upregulates or induces PGS<sub>2</sub>. One report suggests that insulin enhances IL-1-induced PGS<sub>2</sub> expression in renal mesangial cells but has no effect by itself (47). Some report that insulin infusions that induce hypoglycemia upregulate PG metabolism in the brain (48), whereas others reports suggest that insulin has little effect on vascular PG production (49), or that its deficiency actually increases PG production (50). In this study, we did not find evidence that insulin injection induces PGS<sub>2</sub> expression, as monocytes of PGS<sub>2</sub><sup>+</sup> high-risk subjects did not become positive after the initiation of subcutaneous insulin. Furthermore, we found that monocytes of insulin-treated type 2 diabetics expressed PGS<sub>2</sub> at control levels (data not shown). Finally, a significant percentage of the moderate-risk and AI groups expressed high levels of PGS<sub>2</sub> but were not treated with insulin. Together, these data suggest that insulin treat-

**Figure 2**

Percentage of CD14<sup>+</sup> monocytes expressing PGS<sub>2</sub> is significantly higher in subjects at risk for IDDM compared with controls. Data from blood samples taken from subjects at risk for IDDM, relatives, and controls. The first 3 data sets (Controls, Relatives, All Subjects) are data taken from all participants in the study. "All Subjects" data are divided into groups based on risk (i.e., low-, moderate-, and high-risk IDDM subgroups; established diabetics [IDDM]; and ICA<sup>+</sup> individuals with clinically established AI). The percentages of PGS<sub>2</sub><sup>+</sup>/CD14<sup>+</sup> monocytes from relatives and all subjects were significantly different from the normal control group, as assessed by weighted Tobit analysis (*P* = 0.004). Solid lines indicate group means; the dotted line represents the control group mean plus 2 SDs, the level used to define high-level PGS<sub>2</sub> expression (9.9%).



**Table 1**Flow cytometric analysis of activation antigens in CD14<sup>+</sup> monocytes

	PGS <sub>2</sub>	TNF- $\alpha$	IL-10	Pan-DR	CD69	CD105
Controls	2.1 $\pm$ 2.6 <i>n</i> = 55 (24)	5.9 $\pm$ 6.7 <i>n</i> = 23(14)	1.7 $\pm$ 1.9 <i>n</i> = 10 (8)	18.8 $\pm$ 20.2 <i>n</i> = 14(13)	29.0 $\pm$ 25.1 <i>n</i> = 19(14)	5.1 $\pm$ 6.6 <i>n</i> = 9 (9)
Pre-IDDM subjects	11.3 $\pm$ 15.9% <i>n</i> = 112 (70)	3.1 $\pm$ 3.7 <i>n</i> = 24 (24)	1.6 $\pm$ 4.1 <i>n</i> = 10 (10)	21.1 $\pm$ 19.6 <i>n</i> = 19 (19)	22.4 $\pm$ 21.1 <i>n</i> = 23 (22)	7.6 $\pm$ 6.1 <i>n</i> = 6 (6)
Significance difference	<i>P</i> = 0.004 <sup>A</sup>	NS	NS	NS	NS	NS
Statistical analysis	Weighted Tobit analysis	Student's <i>t</i> test	Student's <i>t</i> test	Student's <i>t</i> test	Student's <i>t</i> test	Student's <i>t</i> test

Values represent mean percentage  $\pm$  SD; *n* = the number of samples analyzed (number in parentheses indicates number of individuals sampled). Freshly isolated PBMCs were examined for the expression of PGS<sub>2</sub>, intracellular and surface TNF- $\alpha$ , intracellular IL-10, and surface DR, CD69, and CD105 on CD14<sup>+</sup> monocytes by dual-color flow cytometry. For each of the activation antigens analyzed other than PGS<sub>2</sub>, no significant statistical significant difference (NS) was found between controls and subjects (Student's *t* test). <sup>A</sup>

ment is not responsible for the increased percentage of subjects expressing PGS<sub>2</sub> in the high-risk group.

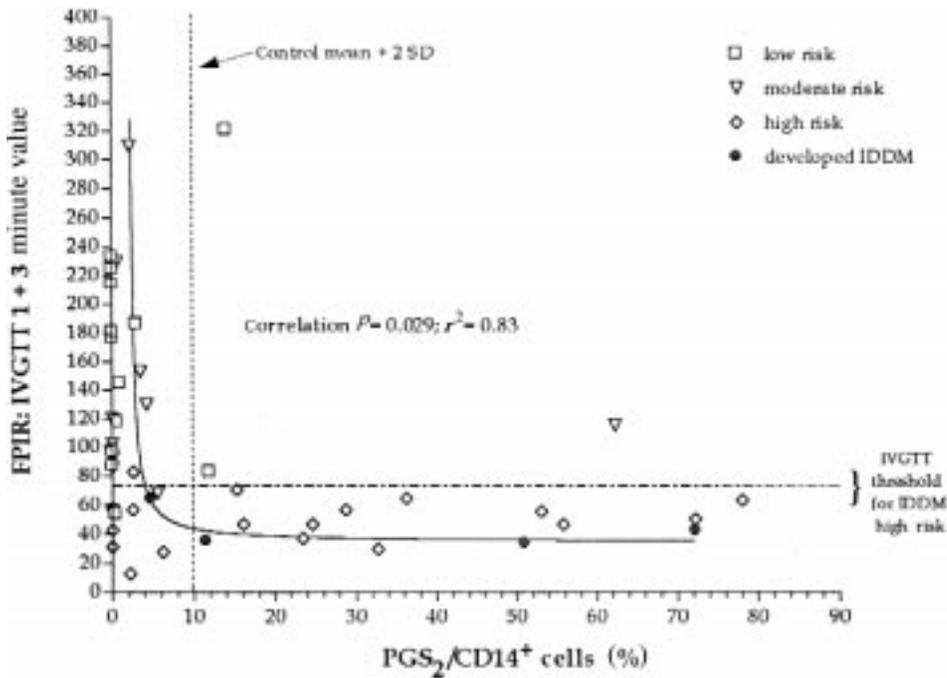
Another factor known to influence PG metabolism is hyperglycemia (51). Subjects in the high-risk group were monitored for glucose intolerance and progression toward diabetes by standard OGTT and hemoglobin A1C periodically during clinical visits. None of the subjects tested in our PGS<sub>2</sub> analysis sample group were diabetic by OGTT at the time of PGS<sub>2</sub> analysis. Of the 30 subjects analyzed, 13 had OGTT and 14 had hemoglobin A1C data taken during the same visit when PGS<sub>2</sub> expression was analyzed. Four of the 13 subjects were found to be glucose intolerant by OGTT, and 2 of these 4 expressed PGS<sub>2</sub> levels above those of controls. There was no correlation of PGS<sub>2</sub> expression with glucose levels at any point in the OGTT time course (fasting to 2 hours after glucose ingestion;  $\tau$ -b = -0.09; *P* = 0.20). Likewise, we found no correlation of PGS<sub>2</sub> expression with hemoglobin A1C data available on 14 of our subjects ( $\tau$ -b = 0.13; *P* = 0.86). We also assessed the effect of hyperglycemia on PGS<sub>2</sub> expression in vivo by analyzing PBMCs from hyperglycemic and nonhyperglycemic type 2 diabetics. None of the 8 type 2 diabetic individuals tested expressed PGS<sub>2</sub> levels greater than those seen in controls, regardless of their blood glucose levels at the time of testing (range: 92–271 mg/dL; mean: 176.1 mg/dL  $\pm$  SD 56.65). These data suggest that the aberrant PGS<sub>2</sub> expression seen in the at-risk subjects is independent of the glyceemic state of the individual.

*Monocytes of subjects at risk for IDDM are not activated.* Because PGS<sub>2</sub> is an early response gene and its expression is normally indicative of monocyte activation (28), we examined these cells for multiple activation markers by flow cytometry. The mean percentage and the mean fluorescence intensity of monocytes expressing pan-DR, CD69, intracellular TNF- $\alpha$  and IL-10, and CD105 were not significantly different in ICA<sup>+</sup>/PGS<sub>2</sub><sup>+</sup> subject cells relative to unstimulated ICA<sup>-</sup>/PGS<sub>2</sub><sup>-</sup> control cells (Table 1). When monocytes from both control subjects and those at risk for IDDM were activated with LPS, however, they expressed high levels of CD69, CD105, DR, and TNF- $\alpha$  in addition to PGS<sub>2</sub> (data not shown). Because we have little supportive evidence for activation, aberrant PGS<sub>2</sub> expression may occur secondary to intrinsic defects in the regulation of this enzyme. To date, we have not found evidence for polymorphism in the PGS<sub>2</sub> gene to

account for its dysregulation. We are currently investigating factors that regulate this enzyme, including IL-10, a potent suppressor of PGS<sub>2</sub> expression (52). Our preliminary findings indicate that PGS<sub>2</sub> expression is resistant to IL-10 regulation in approximately 50% of subjects at risk for IDDM.

*PGS<sub>2</sub> expression correlates with clinical markers of high risk for IDDM.* Because PGS<sub>2</sub> expression was more prevalent in IDDM-prone subjects, we examined correlations between PGS<sub>2</sub> expression and known IDDM risk factors, e.g., HLA DR03/04 or DQ $\beta$ 0201/0302, autoantibodies, and low FPIR. High-level PGS<sub>2</sub> expression is predominant in ICA<sup>+</sup> individuals — especially those carrying the HLA alleles DR03/04, DQ $\beta$ 0302, or DQ $\beta$ 0201 — although this relationship was not statistically significant. We found, however, that PGS<sub>2</sub> expression does show a strong inverse correlation with FPIR (*P* = 0.0201; correlation of maximum PGS<sub>2</sub> values of 46 subjects; *r*<sup>2</sup> = 0.83) (Figure 3). Most high-risk subjects underwent FPIR testing several months before PGS<sub>2</sub> determination, resulting in variable time lags between FPIR and PGS<sub>2</sub> analyses. To account for this variability, an ROC curve analysis with time-weighted peak values for PGS<sub>2</sub> expression was performed. This analysis suggests that PGS<sub>2</sub> protein expression correlated inversely with insulin levels and may be predictive of a low FPIR (Figure 4). An ROC analysis of a larger set of data with elimination of the time differential, however, is needed to firmly establish PGS<sub>2</sub> expression as a predictor of risk for IDDM. These initial data suggest that among autoantibody-positive subjects, PGS<sub>2</sub> expression identifies individuals with low insulin secretory reserve and a high risk for IDDM.

*Prostanoids produced by PGS<sub>2</sub> inhibit CD25 expression on T cells.* Because PG markedly suppress lymphocyte activation (27), we postulated that heightened PG production by monocytes expressing PGS<sub>2</sub> may inhibit this process in T cells. To test this hypothesis, we assessed CD25 expression on CD3<sup>+</sup> T cells of PBMCs from at-risk PGS<sub>2</sub><sup>+</sup> subjects and healthy controls activated with PHA (5  $\mu$ g/mL) in the presence and absence of the PGS<sub>2</sub>-specific inhibitor NS398 (5  $\mu$ M). When activated by PHA, PGE<sub>2</sub> production by PBMCs from subjects at risk for IDDM was 2-fold higher than that of controls (977.3  $\pm$  246.1 [*n* = 23] vs. 429.7  $\pm$  115.5 pg/10<sup>6</sup> cells [*n* = 8]; *P* = 0.05) and was reduced to very low levels in both groups (0–150 pg/10<sup>6</sup> cells) by NS398. It has previously been



**Figure 3**

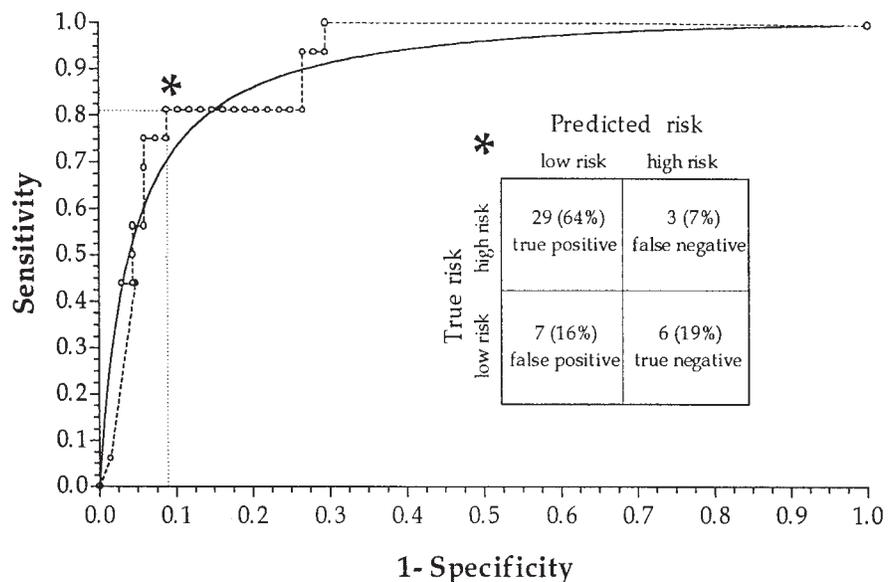
PGS<sub>2</sub> expression in CD14<sup>+</sup> monocytes correlates with FPIR, a clinical criterion for IDDM risk assessment. PBMCs from subjects at low, moderate, and high risk for IDDM were analyzed for PGS<sub>2</sub> expression in CD14<sup>+</sup> monocytes by flow cytometry. The “Developed IDDM” designation in this data set indicates 4 high-risk-group individuals who developed diabetes during the period of observation. The correlation of maximum PGS<sub>2</sub> levels obtained during the observation period with insulin secretory capacity (FPIR) was determined using Prism 2.01 software (Graph Pad Software for Science Inc., San Diego, California, USA). A significant inverse correlation between insulin secretion and PGS<sub>2</sub> expression was found ( $P = 0.0201$ ;  $n = 46$  total subjects), with best fit being a hyperbolic curve ( $r^2 = 0.83$ ).

shown that PGS<sub>1</sub> is expressed in monocytes; therefore, the reduction of PGE<sub>2</sub> to very low levels by treatment with NS398 in our experiments suggests that PGS<sub>1</sub> may be responsible for the residual PGE<sub>2</sub> production (24, 26, 32, 43). Alternatively, the concentration of NS398 used may have been inadequate to block all PGS<sub>2</sub> activity. The marked reduction of PGE<sub>2</sub> production by NS398, a

PGS<sub>2</sub>-specific inhibitor, strongly suggests that the bulk of PG is produced by PGS<sub>2</sub>.

Inhibiting PGS<sub>2</sub> activity during PHA activation significantly increased CD25 expression on CD3<sup>+</sup> T cells from PGS<sub>2</sub><sup>+</sup> subjects at risk for IDDM subjects ( $2.0 \pm 0.1$ -fold;  $n = 23$ ), but not in controls ( $0.45 \pm 0.37$ -fold;  $n = 9$ ) (Figure 5;  $P = 0.04$ , Student’s  $t$  test). Flow cytometric analysis

C = 0.90



**Figure 4**

ROC curves derived from PGS<sub>2</sub>/CD14 expression and FPIR comparison. ROC analysis was used to examine the property of positive PGS<sub>2</sub> expression (>9.9%) as a predictor of low FPIR. The analysis includes a correction for the variation in time between PGS<sub>2</sub> and FPIR analyses. The resulting curve was generated with a c-statistic (area under the curve) of 0.9. An example of the ability of PGS<sub>2</sub> expression to predict a low FPIR is given in the inset table, with a decision threshold set at 0.81 (\*).

of T-cell subsets demonstrated that blocking PGS<sub>2</sub> significantly increased CD25 expression on pre-IDDM CD8<sup>+</sup> T cells when compared with control CD8<sup>+</sup> T cells ( $P = 0.04$ ; Student's  $t$  test). Although CD25 expression on CD4<sup>+</sup> T cells of subjects at risk for IDDM increased in a similar fashion, no statistical difference from that of control T cells was found ( $P = 0.29$ ).

Because PGs can also inhibit IL-2 production, we also examined this cytokine in the supernatants of PHA-activated PBMCs by ELISA (Figure 5). NS398 increased PHA-stimulated IL-2 production in 3 of 9 control individuals tested ( $n = 9$ ; mean fold change =  $1.2 \pm \text{SD } 0.75$ ). In PBMCs of subjects at risk for IDDM, NS398 treatment caused an increase ( $n = 16$ ; mean fold change =  $53.6 \pm \text{SD } 143.3$ ) in IL-2 production in 11 of 16 subjects examined. This effect was not universal, and was less dramatic than the effects seen on CD25 expression. Interestingly, in the subjects that increased IL-2 production in the presence of NS398, there was a parallel increase in CD25 expression. These data suggest that high-level PGS<sub>2</sub> expression in monocytes suppresses IL-2 signal transduction critical for the activation of T cells.

Because non-MHC factors may interact with MHC susceptibility alleles to compound APC dysfunction, we examined the combined contribution of MHC class II antigens and PGS<sub>2</sub> to CD25 expression in subjects at risk for IDDM. When examined in this context, blocking PG production significantly increased T-cell CD25 expression in subjects expressing the IDDM susceptibility alleles *DR04/DQ0302* compared with individuals at risk for IDDM carrying other HLA alleles ( $P = 0.002$ , ANOVA) (Figure 5). These data suggest that the diabetes susceptibility alleles interact with PGS<sub>2</sub> expression to limit T-cell activation.

## Discussion

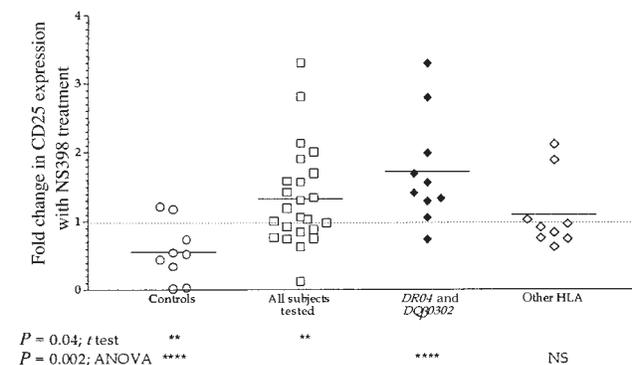
Our studies in the NOD mouse, and now in humans, support the central role of APCs in the development of IDDM. In this report, we detail an APC defect, constitutive PGS<sub>2</sub> expression, in established type 1 diabetics and in individuals at higher genetic, immunological, and familial risk for this disease (compared with the control population). Aberrant PGS<sub>2</sub> expression is enriched in individuals at the greatest risk for IDDM, i.e., those with low insulin secretory reserve. Thus, flow cytometric analysis of PGS<sub>2</sub> expression may prove to be an effective screening tool for assessing IDDM risk, and may identify a cohort of individuals with a high probability of progressing to diabetes. Long-term prospective studies are currently under way to test PGS<sub>2</sub> expression as a predictor of autoantibody production and progression to diabetes.

Furthermore, we found that the inhibition of PGS<sub>2</sub> enzymatic activity enabled increased expression of CD25 and IL-2 by PHA-activated T cells, suggesting that the PGS<sub>2</sub>-mediated PG production disrupts normal T-cell activation. These findings suggest that high-level monocyte PGS<sub>2</sub> expression contributes to the defect in IDDM APC activation of T cells through its negative effects on IL-2 signaling. The limitation in T-cell activation caused by PGS<sub>2</sub> was most profound in subjects expressing the IDDM MHC class II susceptibility alleles *DR04* and *DQ0302*. These studies suggest that PGS<sub>2</sub>, in conjunc-

tion with high-risk MHC class II antigens, may limit T-cell activation and IL-2 signaling.

The role of IL-2 signaling in the maintenance of T-cell tolerance has been recently examined using IL-2, CD25 (IL-2R $\alpha$ ), IL-2R $\beta$ , and IL-2R $\gamma$  knockout mice (53–56). Their studies demonstrate that IL-2 signaling is essential for induction of AICD in T cells (54, 55). High-level IL-2 signaling primes T cells for AICD via its upregulation of FasL transcription and inhibition of FLIP, the inhibitor of FLICE (caspase 8), essential for initiation of the caspase cascade (56, 57). The difference between high and low levels of IL-2 signaling in activated T cells appears to center on the upregulation of CD25, which allows the high-affinity binding of available IL-2 and enhancement of IL-2 signal transduction. It may be that this essential difference in IL-2 signaling delineates outcomes of T-cell activation, whereby high levels of activation lead to regulatory cells, and lower levels of IL-2 signaling promote the activation of effector cells. Evidence from CD25 knockout mice has hinted at this possibility, but definitive evidence is still lacking (58).

Our data raise several questions regarding the natural history and role of PGS<sub>2</sub> expression in the pre-IDDM phase. For example, does monocyte activation and PGS<sub>2</sub> expression arise secondary to a phase of the



**Figure 5**

NS398 inhibition of PGS<sub>2</sub> enzymatic activity affects T-cell expression of CD25 during PHA activation. PBMCs were cultured in the presence of PHA (5  $\mu\text{g/mL}$ ) with or without NS398 (5  $\mu\text{M}$ ) for 24 hours. The expression of CD25 on CD3<sup>+</sup> T cells was determined by dual-color flow cytometry. “All subjects tested” includes ICA<sup>+</sup> diabetics, ICA<sup>+</sup> moderate-risk subjects, ICA<sup>+</sup> high-risk subjects, and those classified as AI. Baseline values for the percentage of CD25<sup>+</sup>/CD3<sup>+</sup> cells in freshly isolated PBMCs were not significantly different between the groups assayed (controls:  $4.61 \pm \text{SD } 4.6$ ,  $n = 12$ ; other HLA subjects:  $6.2 \pm \text{SD } 6.7$ ,  $n = 10$ ; and *DR04/DQ0302*:  $4.5 \pm \text{SD } 3.9$ ,  $n = 14$ ). The data are presented as a fold increase, calculated as the percent of CD3<sup>+</sup> T cells expressing CD25 when activated with PHA in the presence of NS398, relative to CD25 expression on CD3<sup>+</sup> T cells activated with PHA alone. For individuals with multiple sample runs, the fold increase of each assay run was averaged to give a mean fold increase shown here. Statistical analysis demonstrated a significant difference between controls and all subjects ( $P = 0.04$ ; Student's  $t$  test). When HLA data were available, the CD25 data were also analyzed by this parameter. Enhancement of T-cell CD25 expression was most prevalent in subjects expressing both *DR04* and *DQ0302* ( $P = 0.012$ ; 1-way ANOVA), when compared with other subjects expressing HLA DR alleles 16, 03, 01, 07, 13a, and 08 and DQ $\beta$  alleles 05, 0201, 0303, 0604, and 04.

autoimmune process wherein a crescendo of Th1 responses and heightened IFN- $\gamma$  production accelerates  $\beta$ -cell destruction? Our data do not support this hypothesis, as monocytes of subjects at risk for IDDM do not express levels of activation antigens above those of PGS<sub>2</sub> controls. Alternatively, an intrinsic, perhaps genetic, monocyte defect affecting the regulation of PGS<sub>2</sub> expression may contribute to the autoimmune process leading to IDDM. If intrinsic monocyte defects are responsible for aberrant PGS<sub>2</sub> expression in pre-IDDM humans, expression of high levels of this cyclooxygenase may identify subjects at high risk, regardless of the phase of their disease. Our preliminary studies have found that PGS<sub>2</sub> expression precedes detectable autoantibodies in very young infants with genetic risk and a family history of diabetes (S.A. Litherland, unpublished data). These data suggest that PGS<sub>2</sub> is present before the development of standard markers for autoimmunity (e.g., autoantibodies), and support the concept that there is dysregulation of this enzyme very early in the autoimmune process, perhaps on the basis of an intrinsic monocyte defect.

Our findings demonstrate that aberrant constitutive PGS<sub>2</sub> expression in monocytes defines a defect in this APC subpopulation, which is a novel risk marker for IDDM. If PGS<sub>2</sub> is confirmed to play a direct role in the immunopathogenesis of human IDDM, the use of PGS<sub>2</sub>-specific inhibitors may constitute a new pharmacological approach for the prevention and treatment of this disease.

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