CD247, a Novel T Cell–Derived Diagnostic and Prognostic Biomarker for Detecting Disease Progression and Severity in Patients With Type 2 Diabetes Mellitus

OBJECTIVE
We have previously shown that chronic inflammation results in immunosuppression associated with CD247 downregulation in T lymphocytes. Type 2 diabetes mellitus (T2DM) is known to be associated with chronic inflammation. We therefore sought to examine CD247 expression levels in patients with T2DM and to assess whether it can serve as a diagnostic and prognostic biomarker for disease complications and outcomes.

RESEARCH DESIGN AND METHODS
Peripheral blood samples from 75 T2DM patients and 40 healthy control subjects were collected and analyzed for the expression level of CD247 in T lymphocytes. Subjects with T2DM underwent a medical interview with physical examination and were followed for an additional average of 19.2 ± 0.9 months to determine the occurrence of major adverse disease end points. The relationship between the level of CD247 expression and disease status at the time of blood draw and the ability of the marker to identify future complications was evaluated.

RESULTS
We observed a significant reduction in CD247 expression levels in T lymphocytes of T2DM patients when compared with healthy volunteers. CD247 downregulation was associated with disease severity, complications, and the occurrence of future cardiovascular events, suggesting its potential use not only as a diagnostic but also as a prognostic biomarker.

CONCLUSIONS
Our results suggest the use of CD247 as a biomarker in diabetic patients for evaluating the state of chronic inflammation that contributes to morbidity and mortality in this disease and for the prediction of future cardiovascular events.
found to be significantly elevated in diabetic patients with existing coronary artery disease (2–4), diabetic nephropathy (3,5–7), and retinopathy. However, neither of these parameters is indicative of chronic inflammation or the patients’ immune functionality. For example, conflicting results exist as to the correlation between CRP and diabetes-related microvascular complications and its usefulness as a biomarker of disease progression (8). Additionally, the lack of CRP specificity regarding its ability to distinguish between acute and chronic inflammation and susceptibility to the effects of certain anti-inflammatory medications such as statins and aspirin (4), frequently used in diabetic patients, may diminish its usefulness in clinical practice.

We have previously shown that chronic inflammation leads to T-cell immunosuppression associated with CD247 downregulation (9). CD247 (also referred to as the ζ-chain) is part of the T-cell antigen receptor (TCR) complex, which plays a key role in receptor expression and signaling leading to optimal effector T-cell functions (10,11). Interestingly, in T cells, the observed immunosuppression is associated with a unique CD247 downregulation while the remaining TCR subunits are unaffected. This phenomenon characterizes chronic inflammation and does not occur during an acute inflammatory response (9,12). Moreover, neutralization of the chronic inflammatory environment leads to CD247 expression recovery and immune function recuperation to normal levels, indicating a reversible phenomenon. CD247 downregulation was previously described in chronic inflammatory diseases, including celiac disease (13), chronic obstructive pulmonary disease (14), and systemic lupus erythematosus (15) as well as different types of cancer (9). Thus, CD247 may serve as a potential biomarker for evaluating the immune status in pathologies characterized by chronic inflammation.

Currently, the widely used biomarkers for disease severity and progression in diabetes rely on metabolic parameters and are limited in their ability to improve risk stratification with regards to disease-related outcomes (16). Identifying a more mechanistically relevant biomarker that is linked not only to the metabolic processes but also to the underlying inflammatory process may provide an invaluable clinical tool to predict disease progression and effectiveness of therapeutic interventions.

In this study we hypothesize that the expression levels of T-cell CD247, which is a unique biomarker that senses chronic inflammation and associated immunosuppression, in patients with T2DM are associated with parameters of disease severity and progression. Herein, we present evidence of such an association and suggest a possible clinical use for measurement of CD247 expression levels as a novel biomarker for diabetes-related outcomes.

### RESEARCH DESIGN AND METHODS

#### Subjects

A total of 115 subjects participated in the study (40 healthy subjects and 75 subjects with T2DM). Healthy control subjects were recruited among blood donors in the Hadassah University Hospital blood bank facility. Only healthy donors without comorbidities such as diabetes, heart disease, etc., and who did not receive in a period of 3 weeks before the blood donation any anti-inflammatory drugs, were selected to participate in the experiment. The information was obtained by a detailed medical questionnaire obtained at the time of blood donation. Subjects with T2DM were recruited after signing a written informed consent to participate in the study. The patients underwent a full medical interview with physical examination.

The diagnosis of diabetes and diabetes-related complications was based on medical history obtained during the interview
and medical records available in the Hadassah Hospital database. Seventy-five subjects with diabetes were followed through office visits and medical records for an average of 19.2 ± 0.9 months to determine the occurrence of major adverse disease end points (a composite of death from any cause, cerebrovascular event, ST segment elevation myocardial infarction, non-ST segment elevation myocardial infarction, cardiac catheterization, cardiac artery bypass graft surgery, pulmonary edema with hospitalization, or angina pectoris with hospitalization).

**Results**

**Patient Population**

Seventy-five subjects with T2DM and 40 healthy control subjects were recruited. Mean duration of diabetes was 14.04 ± 9.93 months and mean A1C was 8.53% ± 1.78% (70 ± 14.6 mmol/mol). Of the diabetic subjects, 69% had diabetes-related complications, with 34.7% suffering from cardiovascular disease (CVD), 32% from retinopathy, 36% nephropathy, and 32% neuropathy. A majority of the diabetic subjects were treated with metformin (70.7%), a statin (86.7%), and aspirin (78.7%). When compared with healthy volunteers, subjects with T2DM were generally older and had relatively higher CRP levels (<0.5 mg/dL in healthy subjects vs. 0.86 ± 2.55 in subjects with diabetes). When compared with T2DM subjects without complications, T2DM subjects with complications had a longer duration of disease (15.31 ± 10.29 vs. 7.9 ± 5.6 years, P < 0.001). Numeric differences in A1C and CRP did not reach statistical significance (Table 1).

**Significant Reduction in CD247 Expression Levels in Subjects with T2DM**

CD247 expression levels were determined in T cells from blood samples of control subjects and subjects with T2DM by gating on CD3ε^+CD56^− T cells, as depicted in Fig. 1A. A significant reduction in CD247 expression levels was noted in subjects with T2DM (% CD247 expression 81.8% ± 2.5% in diabetic vs. 100% ± 2.2% in healthy subjects, P < 0.001) (Fig. 1B). To verify that the observed reduction in CD247 expression was not the result of TCR internalization due to acute T-cell activation, a process in which all TCR subunits are downregulated, we measured in all samples CD3ε expression levels as a representative of remaining TCR complex. No apparent differences in CD3ε expression (99 ± 2 in diabetic vs. 98.8 ± 0.8 in healthy subjects, P = 0.462) (Fig. 1C) were observed between healthy subjects and subjects with T2DM. These results indicate a chronic inflammatory environment in subjects with T2DM and associated

**Determination of CD247 Expression**

**Peripheral Blood Collection**

Peripheral blood samples were collected into heparin-vacutainer tubes and were gradually frozen within 6 h in freezing buffer that contained 20% DMSO and 80% FCS. Blood samples were kept frozen until thawed for analysis.

**Flow Cytometry Analysis**

Human whole blood cells were stained using anti-CD247, anti-CD3, and anti-CD56 antibodies, all purchased from Biolegend and used according to the manufacturer’s protocol. For cell surface labeling of CD3 and CD56, cells were incubated 30 min at 4°C with the antibodies and washed with FACS buffer containing 2% BSA and 0.05% NaN3 in PBS. The cells then underwent intracellular labeling of CD247; cells were fixed for 20 min with 1% paraformaldehyde at 4°C, permeabilized for 10 min with 0.1% saponin/PBS at room temperature, and then washed with FACS buffer and labeled by a similar procedure as used for cell surface labeling. Samples were collected in a FACS Calibrator using Cell Quest software (BD) and analyzed using the FCS express software (De Novo Software). Expression of a given molecule per cell was detected by FACS analysis.

**Data Analysis**

The data are presented as the mean ± SEM unless specified otherwise. Statistical analysis of CD247 expression level in different subpopulations and with different diabetes-related complications was performed using Student t test. Event occurrence after measurement of CD247 expression levels was plotted as a Kaplan-Meier cumulative event curve and assessed by log-rank test using SPSS software. In all tests, P < 0.05 was considered statistically significant.

**Figure 1**—Decreased CD247 expression in T cells isolated from whole blood of diabetic vs. healthy control subjects. CD247 and CD3ε expression levels in T cells were analyzed by flow cytometry gating on the CD3ε^+CD56^− cells within the lymphocyte fraction (A). Only the expression level of CD247 (B) but not of CD3ε (C) was significantly reduced in diabetic (DM) vs. healthy control subjects (control). P < 0.0001 and P = 0.462, respectively. NS, not significant; MFI, mean fluorescence intensity.
immunosuppression, as reflected by the unique CD247 downregulation.

**CD247 Expression Is Related to Disease Severity and Complications**

To further characterize whether changes in CD247 expression levels in subjects with T2DM are associated with disease-related complications, we compared its expression levels in subjects with and without diabetes-related complications. Whereas there was only a slight but significant reduction in CD247 expression between diabetic subjects without complications and healthy subjects (% CD247 expression $100 \pm 2.2$ in healthy subjects [control] vs. $90.9 \pm 4$ diabetic subjects without complications [DM], $P = 0.03$) (Fig. 2A), a more prominent and significant reduction in CD247 expression was noted in diabetic subjects with complications (% CD247 expression $77.7 \pm 3$ in diabetic subjects with diabetes-related complications [DM-c] vs. diabetic subjects without complications [P = 0.006] and vs. healthy subjects [P < 0.0001]) (Fig. 2A). When assessing the relation between A1C and complications, we found that the mean A1C value was higher in subjects with diabetes-related complications versus those without ($8.82 \pm 0.27$ [73 ± 1 mmol/mol] for DM-c vs. $8.0 \pm 0.35$ [64 ± 1.3 mmol/mol] for DM, $P = 0.04$) (Fig. 2B).

It is important to note that other disease parameters such as age and CRP did not differ between diabetic subjects with or without complications (age $61.38 \pm 2.72$ years for DM vs. $63.62 \pm 1.37$ years for DM-c, $P = 0.2$; CRP $0.8 \pm 0.12$ for DM vs. $0.92 \pm 0.1$ for DM-c, $P = 0.26$) (Fig. 2C and D). Interestingly, whereas a significant correlation was observed between CD247 expression levels and disease duration ($P = 0.017$), no clear correlation was observed between CD247 expression levels and A1C, CRP, sex, or age (Supplementary Fig. 1). Moreover, no relation between CD247 expression levels and various treatments such as aspirin, statins, and metformin was observed (Supplementary Fig. 2).

To better understand the dynamics of change in CD247 expression in relation to diabetes-related complications, we compared CD247 expression levels to the actual number of diagnosed complications/subject and specific complications. As shown in Fig. 3A, CD247 expression levels significantly decreased as the number of diabetes-related complications increased. Among previously diagnosed individual complications, only in subjects with retinopathy and nephropathy was a significant reduction in CD247 expression noted when compared with all subjects in the cohort without these complications (retinopathy: CD247 expression $86 \pm 3.3$% without retinopathy vs. $74.6 \pm 3.9$% with retinopathy, $P = 0.015$; nephropathy: CD247 expression $87.8 \pm 2.9$% without nephropathy vs. $68.4 \pm 4.3$% with nephropathy, $P < 0.001$). Nevertheless, a trend toward reduced CD247 expression was also observed in subjects previously diagnosed with ischemic heart disease (IHD) (CD247 expression $85.1 \pm 3.2$% without IHD vs. $76.3 \pm 4.7$% with IHD, $P = 0.063$), cerebrovascular accident (CVA) (CD247 expression without prior CVA $82.8 \pm 2.7$% vs. $65.9 \pm 9.7$% with CVA, $P = 0.063$), and diabetic foot ulcer (DFU) (CD247 expression $82 \pm 2.7$% without DFU vs. $69 \pm 5.3$% with DFU, $P = 0.056$) (Fig. 3B).

**CD247 Expression Levels as a Potential Biomarker for Impending Cardiovascular Events**

Based on the results described above, we further analyzed the potential use of CD247 expression levels as a biomarker for predicting the occurrence of a composite end point of inflammatory and atherosclerotic cardiovascular and cerebrovascular events (including death from any cause, cerebrovascular event, ST segment elevation myocardial infarction, non-ST segment elevation myocardial infarction, cardiac catheterization, cardiac artery bypass graft surgery, pulmonary edema with hospitalization, or angina pectoris with hospitalization). Figure 3C presents a Kaplan-Meier plot of disease end point occurrence in the subset of subjects with CD247 expression levels $<70$ or $\geq 70$% at baseline. Cutoffs of 60 and 80% were also tested, but the 70% cutoff was found to better differentiate the two outcome populations. Using the log-rank test, a significant difference ($P = 0.048$) was noted between these two populations, suggesting that a baseline CD247 expression level $<70$% may be predictive...
of future CV events in diabetic patients. This predictive significance of low CD247 expression was also observed in the subset of subjects without a previous diagnosis of macrovascular disease (IHD, CVA, and/or peripheral vascular disease [PVD]), n = 10 subjects with CD247 expression <70% vs. n = 31 subjects with CD247 expression ≥70%, P = 0.016). Reduced CD247 expression is associated with disease progression. A: Reduced CD247 expression levels significantly correlate with cumulative diabetes-related complications (P = 0.005 between the mean CD247 expression and number of diabetes-related complications/subject). B: CD247 expression is significantly reduced in subjects with retinopathy and nephropathy (*, retinopathy [P = 0.015]; **, nephropathy [P < 0.001]). C: Kaplan-Meier plot of disease end points in subjects with CD247 expression level <70 and ≥70%. Disease end points were a composite of the following events: death from any cause, cerebrovascular event, ST segment elevation myocardial infarction, non-ST segment elevation myocardial infarction, cardiac catheterization, cardiac artery bypass graft surgery, pulmonary edema with hospitalization, or angina pectoris with hospitalization. CD247 expression levels were measured at baseline and subjects were followed for an average of 19.2 ± 0.9 months for the occurrence of CVD end points. (n = 75 [n = 24 CD247 <70% and n = 51 CD247 ≥70%], P = 0.048 using the log-rank test). NS, not significant.

CONCLUSIONS

Preventing the development of the ominous micro- and macrovascular complications associated with diabetes necessitates a comprehensive understanding of the interaction between glycemia and vascular inflammatory processes as well as identifying predictive biomarkers linking these seemingly distinct events. We present here, evidence that T cell–specific CD247 expression levels may pose as a sensitive and predictive biomarker for chronic inflammation in diabetes, possibly linking glycemic disease processes with inflammatory outcomes such as CVD and nephropathy. Currently, commonly used biomarkers for disease progression and outcome, such as A1C, assess the glycemic exposure but reveal little as to the actual pathologic inflammatory process underlying the progression of micro- and macrovascular complications (16). This has been recently highlighted by evidence from megatrials such as ACCORD (Action to Control Cardiovascular Risk in Diabetes) and ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation), suggesting a dissociation between changes in A1C upon implementation of intensive antihyperglycemic therapy and progression of macrovascular outcomes (17,18). Likewise, a recent study showed that assessment of A1C values in the context of CVD risk assessment provided little incremental benefit for prediction of CVD risk (16).

In light of these studies, a more process-specific biomarker of the pathologic inflammation in diabetes is needed. A biomarker that may shed light on the level of chronic inflammation is expected to both improve our understanding of the factors contributing to the progression of complications in diabetes and offer a mode for assessing therapeutic efficacy and novel therapeutic targets. Commonly used biomarkers for inflammation, such as CRP, have shown a loose correlation to actual disease outcome and may not be sufficiently specific to the diabetic disease process (8). Moreover, CRP levels fluctuate in successive measurements and respond to both chronic and acute inflammatory process, impeding its validity as a biomarker for chronic processes (19).

It is well established that downregulation of the TCR component CD247 is associated with chronic inflammation-induced immunosuppression, as previously described for various types of cancer, chronic infections (HIV, hepatitis C, and leprosy), and autoimmune diseases (arthritis, contact eczema, and lupus) (9). Thus, it is suggested as a biomarker for evaluating the immune status of patients with pathologies characterized by chronic inflammation. Along these observations, we present evidence of a specific decline in the expression levels of CD247, in patients with T2DM and in patients with a more progressive disease (manifested by the occurrence of diabetes-related complications). Of the specific diabetes complications examined, subjects with nephropathy and/or retinopathy had significantly reduced CD247 expression levels, and a tendency
toward a reduced expression was noted in subjects with diagnosed IHD, prior CVA, and DFU when compared with subjects without these specific complications. The lack of significant correlation with other known inflammatory diabetes complications may be due to an inherent characteristic of the process leading to a reduced expression of CD247 or may be the result of the relatively low number of events/subjects tested. For example, only 5 of 75 subjects tested had a previous diagnosis of cerebrovascular disease. Moreover, this analysis is confounded by the fact that 70% of subjects with complications had more than one complication diagnosed.

Assessment of baseline CD247 expression levels in diabetic patients as a biomarker for a composite endpoint of vascular events revealed a worse outcome in the subgroup of patients with reduced CD247 expression levels. These results underscore a potential predictive value of CD247 measurements to stratify the CVD risk in diabetic patients. Moreover, the significant predictive value of low CD247 expression was maintained in the subset of subjects without a previous diagnosis of macrovascular disease, suggesting a potential use of this biomarker in primary prevention of CVD.

CD247 expression levels did not correlate with other parameters of disease severity such as age, A1C, and CRP. This may suggest that CD247 is less amenable to relatively acute fluctuations in glycemic control (which rapidly affect A1C) or acute inflammation (which rapidly affects CRP) and may be better linked to the hypothesized underlying chronic low-grade inflammatory processes that are thought to contribute to the emergence of diabetes-related complications. Interestingly, the ability of both A1C and low CD247 expression to distinguish between patients with and without diabetes-related complications, and the fact that these two markers did not correlate, raise the possibility that they are indeed affected by different disease pathways. Therefore, a combined approach utilizing these two markers may improve our ability to stratify a patient’s risk of developing diabetes-related complications and enhance the efficacy of primary therapeutic interventions.

The study we present is confounded by its relatively small sample size and short follow-up time. However, our promising results suggest a novel approach to risk stratification in diabetes. Rather than focusing on the typical markers of the diabetic metabolic disorder such as glycemia and lipids, CD247 may shed light on the actual pathologic process leading to end organ damage. Understanding these processes may open a new venue for treatment in diabetes, not only addressing the metabolic disturbances but also optimizing and personalizing treatment to prevent the resultant chronic inflammatory processes that are at its core.

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References
8. Fronczek A, Molgeda P, Safranow K, Piechota W, Majkowska L. Increased concentration of C-reactive protein in obese patients with type 2 diabetes is associated with obesity and presence of diabetes but not with macrovascular and microvascular complications or glycemic control. Inflammation 2011;34:349–357
10. Irving BA, Weiss A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. Cell 1991;64:891–901
Supplementary Figure 1. Association between CD247 expression levels and disease parameters. CD247 expression levels were not associated with changes in CRP (A), A1C (B), age (both in subjects with T2DM (C) and healthy controls (D)). A significant correlation was found between CD247 expression levels and disease duration (E) (p=0.017). CD247 expression levels were similar between male and female subjects (F).
Supplementary Figure 2. Lack of effect of different medications on CD247 expression levels. CD247 expression levels were not significantly affected by treatment with medications commonly prescribed to patients with T2DM (including sulfonylureas, insulin, metformin, roziglitahzone, incretins (GLP-1 analogs and DPP-4 inhibitors), aspirin, and statins).
Supplementary Figure 3. Reduced CD247 expression is associated with disease progression in subjects without a prior diagnosis of a composite of IHD, CVA or PVD. Kaplan–Meier plot of disease end points in subjects with CD247 expression level <70% and ≥ 70% and without a prior diagnosis of ischemic heart disease, cerebrovascular disease or peripheral vascular disease. Disease end points were a composite of the following events: death from any cause, cerebrovascular event, ST segment elevation myocardial infarction, non-ST segment elevation myocardial infarction, cardiac catheterization, cardiac artery bypass graft surgery, pulmonary edema with hospitalization or angina pectoris with hospitalization. CD247 expression levels were measured at baseline and subjects were followed for an average of 21.2 ± 1 months for the occurrence of CVD end points. (n=41, n= 10 subjects with CD247 expression<70% vs. n=31 subjects with CD247 expression ≥ 70%, p=0.016).