TABLE 2
Biological processes for upregulated genes in monocytes of diabetic patients

	Z score	Permute *P* value
MAPP name		
Golgi apparatus	3.383	0.000
Ribosomal proteins	3.691	0.002
Unfold protein binding	2.471	0.026
Intracellular protein transport	2.310	0.029
Enzyme-linked receptor protein signaling pathway	2.175	0.042
Nuclear receptor	2.316	0.043
Gametogenesis	−1.998	0.049

mycin compared with untreated monocytes after >6 h of incubation. Treatment of monocytes with a higher concentration of tunicamycin (5 μg/ml) induced more apoptosis (Fig. 5*A* and *B*), and when monocytes were treated with tunicamycin for 12 h, the activity of the proapoptotic protease, caspase-3, significantly increased (Fig. 5*C*). Treatment with tunicamycin coordinately decreased the expression of BCL-2 (Fig. 5*D*) and increased the expression of the ER stress markers, CHOP and BiP (Fig. 5*E*). These results suggest that ER stress promotes apoptosis of human monocytes.

Next, we investigated how tunicamycin-induced ER stress affected the responsiveness of human monocytes to TLR ligands. Treatment of monocytes with tunicamycin for 6 h did not affect the transcriptional and translational expression of TLR2 and TLR4 (data not shown). As shown in Fig. 6*A–C*, however, the expression of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 was downregulated after stimulation with TLR2 and TLR4 ligands. Furthermore, the production of TNF-α, IL-1β, and IL-6 in media was measured by ELISA and found to decrease after treatment of human monocytes with tunicamycin and after stimulation with TLR2 or TLR4 ligands (Fig. 6*D–F*). However, tunicamycin-induced ER stress did not affect expression after treatment of monocytes with the TLR3 ligand, Poly (I:C) (data not shown).

DISCUSSION

In the present study, we observed that PBMCs from patients with diabetes were more susceptible to apoptosis compared with PBMCs from healthy volunteers and that

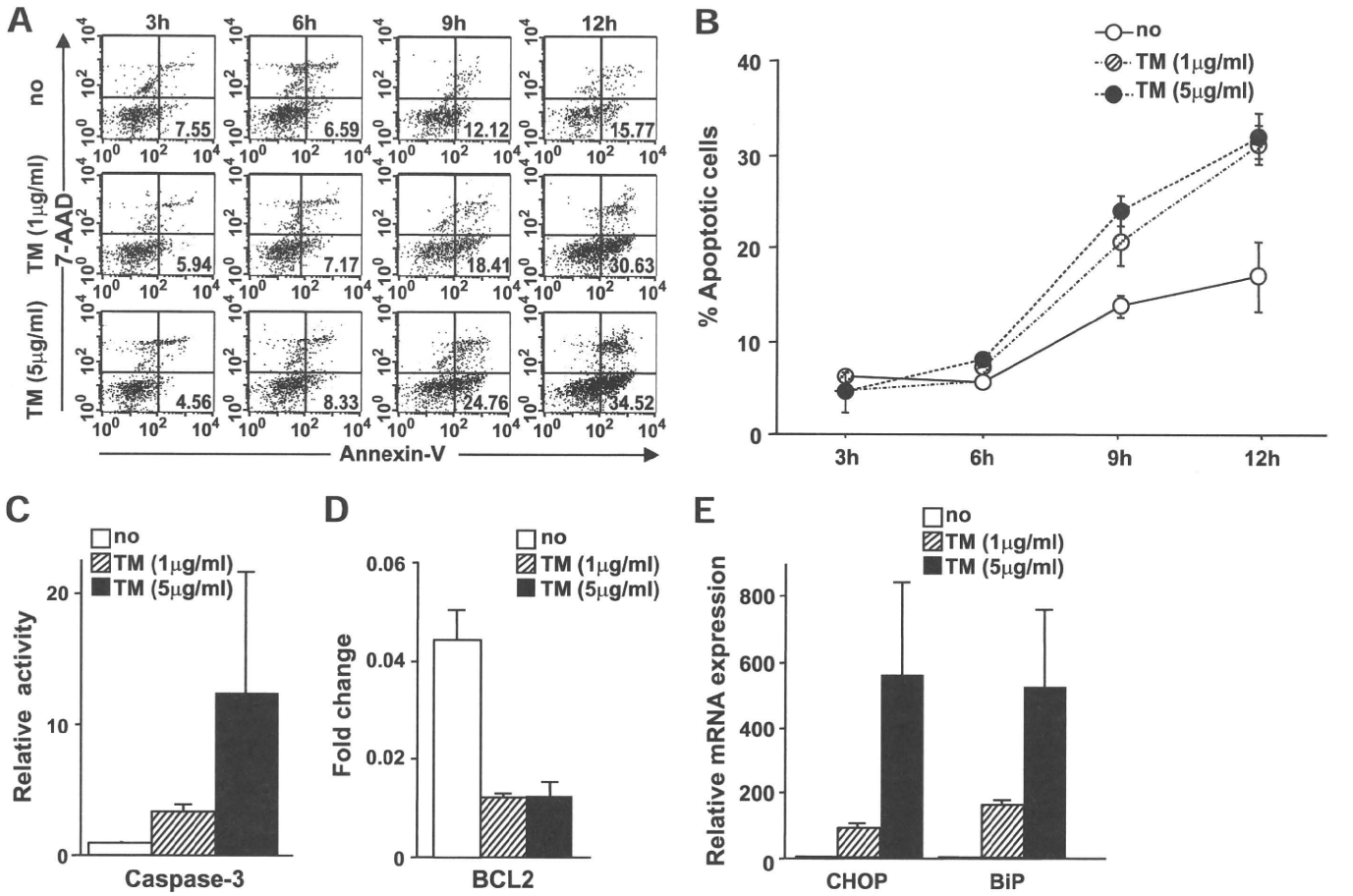

FIG. 5. ER stress enhanced the susceptibility of human monocytes to apoptosis. *A* and *B*: Human CD14$^+$ monocytes obtained from a healthy volunteer were incubated in AIM-V culture media supplemented with tunicamycin (TM) (1 or 5 μg/ml). The frequency of apoptotic cells was analyzed by flow cytometry every 3 h for 12 h. More apoptotic cells were observed among monocytes treated with tunicamycin for >6 h of incubation, compared with untreated monocytes. *A*: Representative scattergram of annexin-V and 7-AAD for monocytes treated with tunicamycin. The numbers in each quadrant indicate the percentage of apoptotic cells. *B*: Apoptotic cells were assessed in triplicate for each condition. Data are expressed as means ± SEM. *C*: Caspase-3 activity in monocytes treated with tunicamycin increased significantly at 12 h of incubation. *D*: The BCL-2 expression in monocytes incubated with tunicamycin for 12 h was downregulated. *E*: The expression levels of the ER stress markers CHOP and BiP in monocytes incubated with tunicamycin for 12 h were significantly upregulated. Data are expressed as means ± SEM of three independent experiments. □, No treatment; ▨, treatment with tunicamycin (1 μg/ml); ■, treatment with tunicamycin (5 μg/ml).

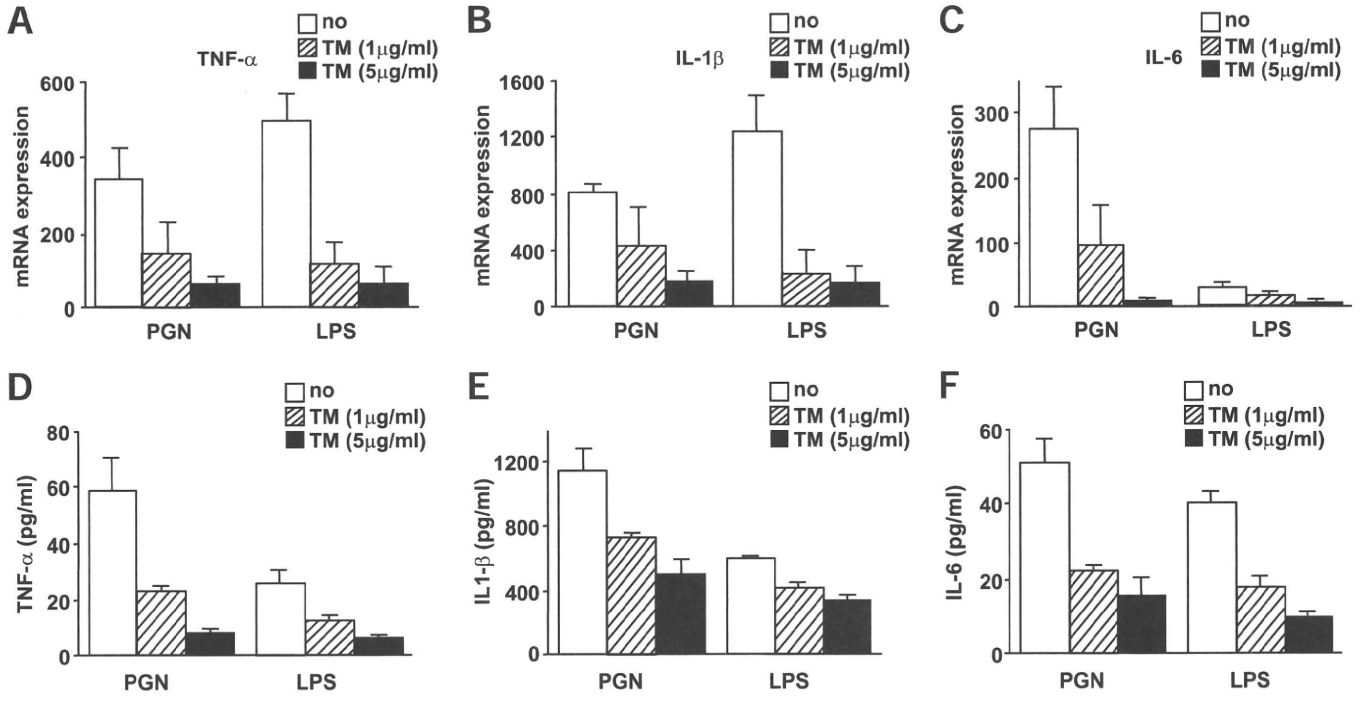

FIG. 6. Expression of proinflammatory cytokines in response to TLR ligand stimuli decreased in human monocytes treated with tunicamycin (TM). Isolated human CD14$^+$ monocytes were incubated in AIM-V culture media with tunicamycin (1 or 5 μg/ml) and stimulated using TLR ligands, PGN, and LPS for 6 h. *A–C*: RTD-PCR analysis showed that the expression of TNF-α (*A*), IL-1β (*B*), and IL-6 (*C*) was downregulated in human CD14$^+$ monocytes treated with tunicamycin, especially at the higher concentration (5 μg/ml). *D–F*: ELISA showed that the production of TNF-α (*D*), IL-1β (*E*), and IL-6 (*F*) in culture media decreased in human monocytes treated with tunicamycin, especially at the higher concentration (5 μg/ml). Data are expressed as means ± SEM of four independent experiments. □, No treatment; ▨, treatment with tunicamycin (1 μg/ml); ■, treatment with tunicamycin (5 μg/ml).

CD14$^+$ monocytes comprised the primary PBMC subpopulation undergoing apoptosis. We also found that CD14$^+$ monocytes from patients with diabetes were hyporesponsive to TLR ligands and that they had attenuated phagocytotic activity. Transcriptional analysis and electron microscopy revealed the presence of ER stress in the affected diabetic monocytes. Consistently, monocytes isolated from nondiabetic patients showed a similar increase in apoptosis and a weakened response to TLR ligands, when they were treated with tunicamycin, indicating that ER stress may be a pivotal mechanism underlying the decreased immunologic function observed in patients with diabetes.

As innate immune-defense mediators, monocytes are capable of ingesting exogenous pathogens to protect the host from infectious diseases. Previous studies have shown that phagocytosis in diabetic neutrophils and monocytes is attenuated (10,11). Similarly, in our study population, monocytes from patients with diabetes were less capable of phagocytosing *E. coli* pathogens compared with monocytes derived from healthy volunteers. This novel finding might explain, at least in part, the decrease in immune function characteristic of patients with diabetes (16). Nevertheless, the detailed mechanisms underlying diabetes-induced decreases in phagocytotic activity remain unclear, because simple high-glucose concentration neither affected the phagocytotic activity and TLR expression nor induced ER stress in nondiabetic monocytes in vitro (data not shown).

The TLRs are pattern-recognition receptors that are important for recognizing pathogens, inducing proinflammatory responses, and preventing the host from acquiring infectious diseases (17–20). The expression of TLR2, TLR3, and TLR4 in CD14$^+$ monocytes was similar between patients with diabetes and healthy volunteers. The administration of a high dose of insulin downregulates TLR expression (21). Transformed monocyte-lineage blastoma cells showed increased TLR expression under hyperglycemic conditions in vitro (22). Type 2 diabetes is characterized as a state of inadequately controlled glycemia associated with hyperinsulinemia due to peripheral insulin resistance (1). Taken together, the TLR expression may be affected by hyperglycemia and hyperinsulinemia in a complex manner. In contrast to the previous finding that monocytes from patients with diabetes were hypersensitive to the TLR ligand, LPS (23,24), we observed that the TNF-α and IL-1β expression from monocytes derived from patients with type 2 diabetes diminished after exposure to PGN, Poly I:C, and LPS—ligands of the TLR2, TLR3, and TLR4 receptors, respectively. These data suggest that diabetes perturbs signaling downstream of the TLRs. In this study, we collected CD14$^+$ monocytes from PBMCs via enrichment using magnetic beads; this protocol was used to remove T-cells, NK cells, B-cells, dendritic cells, and basophils from the PBMC mixture. This is in contrast to the methodology used to isolate these cells in many other studies, in which monocytes were obtained as adherent cells in the culture dish or by a rosetting technique (25,26). CD14$^+$ cells have been shown to be composed of multiple subtypes of activated states; the classical monocyte-isolation methods used in the other studies might unknowingly remove the fraction of monocytes that are susceptible to apoptosis (27). More than half of the CD14$^+$ diabetic monocytes isolated in this study were dead after 12-h incubation, even in media containing physiological concentration of glucose (data not shown).

Our current data showing attenuation of TLR responsiveness to ligands in diabetic monocytes suggest that initial immune responses that are normally triggered by viruses, bacteria, and parasites could be impaired in diabetes, which is consistent with epidemiologic data showing a high incidence of infection in patients with diabetes (3–5).

Gene expression and electron microscopic analysis of monocytes derived from patients with diabetes showed active signatures of ER stress; this is important because ER is an organelle essential for the proper folding and glycosylation of proteins after protein synthesis (28). When cells are under ER stress, protein kinase R–like ER kinase, inositol-requiring enzyme 1, and activating transcription factor 6 are activated and function in the adaptation to stress, proper folding of proteins, and removal of harmful unfolded proteins, respectively (29,30). However, prolonged ER stress leads to apoptotic cell death, which is mediated by CHOP (31). CHOP is a crucial and specific molecule for ER stress–induced apoptosis and alters the transcription of the *BCL-2* gene family members (32). The current study showed that diabetic monocytes had increased levels of ER stress–related apoptotic molecules. Moreover, nondiabetic monocytes treated with tunicamycin, an ER stress inducer, underwent apoptosis in a manner similar to monocytes derived from patients with diabetes. From these data, we conclude that ER stress contributes to the susceptibility of diabetic monocytes to apoptosis.

We also observed that tunicamycin-induced ER stress diminished TLR2 and TLR4 signaling without altering expression of TLRs. Tunicamycin induces ER stress by disturbing N-linked glycosylation (33), and previous reports suggest that perturbations in this glycosylation attenuate TLR2 and TLR4 signaling in vitro (34,35). Hence, these data collectively indicate that ER stress may underlie decreases in TLR2 and TLR4 signaling and affect immune function in patients with diabetes.

TLR3 signaling is different from the other TLR signaling pathway; for example, it is independent of MyD88. TLR2 and TLR4 are expressed on the cell surface, whereas TLR3 is expressed in intracellular compartments such as endosomes (13), and its ligands require internalization before signaling occurs. This suggests that disturbances in TLR3 signaling in diabetic monocytes may be due to reasons other than ER stress. Further investigations are needed to elucidate the detailed mechanisms of attenuated TLR signaling in monocytes from patients with diabetes.

ER stress has been shown to be a mainstay of the diabetic condition. Its pathologic importance in diabetes is especially important in pancreatic β-cells, in which glucose toxicity results in ER stress and insufficient insulin secretion (36–38). The current study suggests that monocytes are yet another population of cells vulnerable to hyperglycemia-induced ER stress and dysfunction. Nevertheless, the mechanisms that render pancreatic β-cells and monocytes vulnerable to ER stress in patients with diabetes remain uncertain.

Diabetes is considered a chronic inflammatory disease. Activated macrophages that produce proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are thought to contribute to insulin resistance in muscle and adipose tissues (39,40). Furthermore, the atherosclerotic complications in patients with diabetes have a basis in inflammation; local inflammatory foci in atherosclerotic lesions are commonly composed of foam cells derived from activated macrophages (41,42). Further studies are needed to determine whether different subpopulations of monocyte-derived cells, for example, systemically circulating and locally residing inflammatory cells, are susceptible to hyperglycemia-induced ER stress and dysfunction.

In conclusion, our findings show that $CD14^+$ monocytes are susceptible to ER stress–induced alterations in inflammatory signaling and apoptosis, which may play a role in the decreased immune function observed in patients with diabetes. Further investigations are needed to discern the mechanisms of diabetes-induced ER stress and perturbations in inflammatory signaling in $CD14^+$ monocytes.

ACKNOWLEDGMENTS

No potential conflicts of interest relevant to this article were reported.

We thank Dr. Iseki for valuable advice and critical comments on electron microscopic examination about ER of monocytes.

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