

Multidose Streptozotocin Induction of Diabetes in BALB/c Mice Induces a Dominant Oxidative Macrophage and a Conversion of T_H1 to T_H2 Phenotypes During Disease Progression

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Received 11 April 2005; accepted 23 May 2005

Macrophages (Mp) are implicated in both early and late phases in type 1 diabetes development. Recent study has suggested that a balance between reductive Mp (RMp) and oxidative Mp (OMp) is possible to regulate T_H1/T_H2 balance. The aim of this study is to investigate the redox status of peritoneal Mp and its cytokine profile during the development of autoimmune diabetes induced by multiple low-dose streptozotocin in BALB/c mice. Meanwhile, the polarization of T_H1/T_H2 of splenocytes or thymocytes was also examined. We found that peritoneal Mp appeared as an “incomplete” OMp phenotype with decreased icGSH along with disease progression. The OMp showed reduced TNF- α , IL-12, and NO production as well as defective phagocytosis activity compared to nondiabetic controls; however, there was no significant difference with IL-6 production. On the other hand, the levels of IFN- γ or IL-4 of splenocytes in diabetic mice were significantly higher compared to the control mice. The ratio of IFN- γ to IL-4 was also higher at the early stage of diabetes and then declined several weeks later after the occurrence of diabetes, suggesting a pathogenetic T_H1 phenotype from the beginning gradually to a tendency of T_H2 during the development of diabetes. Our results implied that likely OMp may be relevant in the development of type 1 diabetes; however, it is not likely the only factor regulating the T_H1/T_H2 balance in MLD-STZ-induced diabetic mice.

INTRODUCTION

Macrophages (Mp) play a pivotal role in specific and nonspecific immunity, and the physiological status of Mp may contribute to the overall regulation of the host defense system. A number of studies have showed the functional heterogeneity of Mp with different cytokine propensity or metabolic activities, therefore inducing distinct immune response such as T_H1-type versus T_H2-type (T_H, T helper). Very recently, Murata et al proposed the functional discrimination of two classes of Mp, namely the reductive Mp (RMp) with a high intracellular content of glutathione (icGSH) and oxidative Mp (OMp) with a reduced content [1]. It was found that T_H1/T_H2 balance might be regulated by the altered balance between RMp and OMp through the distinctive production of TNF- α , IL-12, and NO (nitric oxide) versus IL-6 and IL-10. This classification cast light on the potential role

of the predominant RMp or OMp in inflammatory and autoimmune diseases, such as IDDM (insulin-dependent diabetes mellitus) in NOD (nonobese diabetic) mice [2], spontaneous inflammatory bowel disease [3], cancer immunotherapy [4], corneal allograft [5], and aging-related autoimmune disease [6]. To date, the predominant presence of RMp or OMp in the development of multiple low-dose streptozotocin (MLD-STZ) induced diabetes has been little studied.

For our experiments, we used the MLD-STZ animal model. Recently this model has been attractive for its little or no influence of genetic background, as most new cases of type 1 diabetes are sporadic and occur in families with no previous history of diabetes. Furthermore, as the term “latent autoimmune diabetes mellitus in adults” (LADA) has been introduced to describe an important minority of adult-onset patients with diabetes [7], STZ-induced diabetic mice may serve to be a better model of it since diabetes can be initiated at a relatively older age, when animals had all reached maturity, with a negligible weight loss.

Using this murine model, we found that during the development of diabetes, peritoneal Mp consistently skewed to OMp with gradually decreased icGSH.

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The OMP showed reduced TNF- α , IL-12, and NO production, and also defective phagocytosis activity compared to nondiabetic controls, although there was no significant difference with IL-6 production. On the other hand, the levels of IFN- γ or IL-4 of splenocytes in diabetic mice were significantly higher than the controls. The ratio of IFN- γ to IL-4 level was higher at the early stage of diabetes, then declined several weeks later after the occurrence of diabetes, concomitant with the sequential change of T_H1/T_H2 skewing in the thymus. Thus, the aberrant OMP might play a relevant role in the pathological progression of diabetes, however, it is not likely the only factor regulating the T_H1/T_H2 balance in MLD-STZ-induced diabetes mellitus.

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice were initially purchased from Shandong Laboratory Animal Center and bred in our laboratory animal facility. The mice were housed with free access to water and a standard laboratory diet. Diabetes was induced in mice with STZ (Sigma, St Louis, Mo) as described previously [8]. Briefly, STZ was reconstituted in 25 mM sodium citrate (pH 4.5). Injections were made intraperitoneally (40 mg/kg, IP) within 15 minutes of preparation. Mice were treated with five consecutive daily injections. Nonfasting blood glucose levels were measured once a week, following the administration of STZ by a blood glucose monitor drawing blood from the tail vein (Lifescan, Inc, Milpitas, Calif). Mice with a blood glucose of at least 11.1 mmol (300 mg/DL) were induced gradually after 3 weeks following MLD-STZ of BALB/c mice; those that became diabetic within 1 week were used as diabetic group in the experiment ($n = 6$; blood glucose: 15 ± 2.1 mmol; body weight: 28 ± 1.9 g), while those that became diabetic over 4 weeks were used as advanced diabetic group ($n = 6$; blood glucose: 20 ± 2.5 mmol; body weight: 25 ± 2.9 g). Mice given the same amount of 25 mM citrate buffer were used as the control group ($n = 6$; blood glucose: 5 ± 0.4 mmol; body weight: 28 ± 1.5 g). Even to advanced diabetes mice, there was no significant loss in body weight compared with the controls.

Proliferation assay

The thymus cells or spleen cells proliferation assay on stimulation of ConA (Concanavalin A) was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay as previously described [9]. Briefly, single cell suspensions of either thymocytes or splenocytes were prepared and viability was assessed by trypan blue exclusion. Thymocytes (8×10^5 cells/well) or splenocytes (4×10^5 cells/well) were plated in 96-well plates in RPMI-1640 (medium named after Roswell Park Memorial Institute) complete medium and were stimulated with ConA (30 μ g/ml, Sigma) for 20 hours or 44 hours. Sterile MTT solution (5 mg/ml MTT

in RPMI-1640) was then added into the wells and incubated for another 4 hours until purple precipitate was visible. After moving the medium by centrifugation, the converted dye was solubilized with 200 μ L dimethyl sulphoxide, and the absorbance of the converted dye was measured at a wavelength of 490 nm with background subtraction as 630 nm. The stimulation index (SI) is determined by the absorbance with ConA/the absorbance without ConA.

Peritoneal macrophages

Peritoneal cells (PC) were harvested by injecting total 10 mL of an ice-cooled Hanks-10% FBS (fetal bovine serum)-heparin (10 U/mL) solution into the peritoneal cavity of mice. The collected PCs were added to a microplate at $1-3 \times 10^5$ cells/200 μ L RPMI-1640 medium. The adherent cells after a 2-hour incubation were used as resident peritoneal Mp for production of cytokines and NO by culturing for 48 hours.

Determination of intracellular GSH

Peritoneal cells adherent to dishes were collected by D-Hanks (2.5 mmol/L EDTA) and washed 3 times with cold D-hanks buffer. The cell pellet was immediately lysed with ultrasonic, and after centrifugation, some of the supernatants was assayed for the total protein content using the Coomassie protein assay kit (Jiancheng Co, Nanjing, China). Thereafter, 10% sulfosalicylic acid was added to the remained supernatants to precipitate protein. After centrifugation, supernatants were collected for GSH assay. The cellular GSH concentration was assayed using the GSH kit (Jiancheng Co), and the icGSH was determined as mg GSH/g protein.

Nitrite assay

The accumulation of NO₂ was taken as a parameter for nitrite (NO) production. NO production by Mp was measured in supernatants collected after 48 hours of culture. Briefly, cell-free supernatants were incubated with the Griess reagent for 10 minutes at room temperature and absorbance at 550 nm was measured. The concentration of NO₂ was determined by the square linear regression analysis of a sodium nitrite standard that was measured in each experiment.

Measurement of cytokine levels by ELISA

The IL-4, IL-6, TNF- α , and IFN- γ concentration was determined using an ELISA (enzyme-linked immunosorbent assay) kit (Alpha Diagnostic Intl, Inc, San Antonio, Tex) according to the indication of the manufacturer.

Phagocytosis assays

The phagocytosis activity of Mp was measured by the neutral fuchsin uptake method. 0.075% neutral fuchsin solution was added to adherent Mp (5×10^6 cells/mL), and the samples were incubated for 1 hour at 37°C to study phagocytosis. Cells were then subjected to lysis by adding

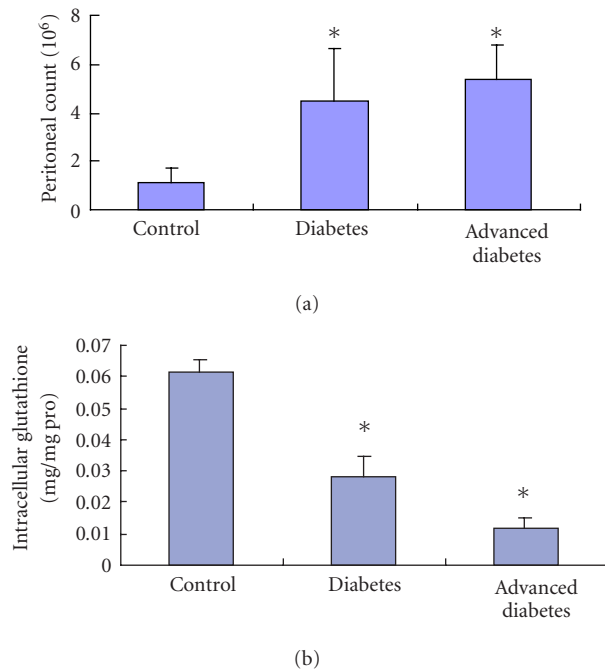


FIGURE 1. The periodic change of peritoneal M ϕ . (a) M ϕ count. There was significant difference between the diabetic and control groups ($P < .05$), and the count increased significantly after the onset of diabetes ($P < .01$). (b) The amount of intracellular GSH in adherent M ϕ of the mice. M ϕ collected from the diabetic mice showed significantly decreased content compared to that of control M ϕ . Results shown are representative of three experiments, and the M ϕ were all resting M ϕ .

lysis solution (0.1 N acetic acid: ethanol = 1: 1, v/v) after washing 3 times to get rid of externally red dye. The absorbance of the cell lysis at 570 nm was measured, and the phagocytosis activity is determined by A value.

Statistical analysis

The results of icGSH levels, NO content, macrophage phagocytosis, ConA-stimulated index, and cytokine levels were expressed as mean \pm SE. This data was analyzed by Student's t test, and the difference was judged at $P < .05$.

RESULTS

The M ϕ count was significantly increased in the peritoneal cavity of diabetic mice ($4.5 \pm 2.21 \times 10^6$ cells/mouse) as compared to nondiabetic controls ($1.1 \pm 0.59 \times 10^6$ cells/mouse) ($P < .05$), and the M ϕ continued to increase 4 weeks after the incidence of diabetes ($5.4 \pm 1.46 \times 10^6$ cells/mouse) (Figure 1a). In addition, the redox status of M ϕ , indexed by icGSH, was significantly decreased in either early (28 ± 6.6 mg/g protein) or advanced diabetic mice (12 ± 3.3 mg/g protein) as compared to the controls (62 ± 3.4 mg/g protein) (Figure 1b). Significant difference was also found between the (early) diabetic mice and the advanced

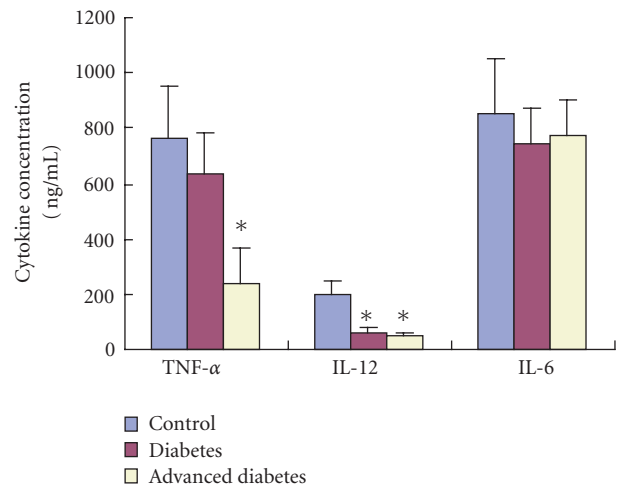


FIGURE 2. Graphical representation of cytokine quantitation (TNF- α , IL-12, and IL-6) in conditioned medium from M ϕ cultures (10^6 cells/mL) on *in vitro* stimulation of LPS (40 mg/L) after 36 hours. The graph is generated from four separate experiments. There were significant differences between control and experimental groups. Note that all cytokines of M ϕ except IL-6 exhibited a significant reduction in diabetic mice.

diabetic ones. Therefore, along with the diabetic progression, the peritoneal M ϕ showed OMP phenotypes with gradually decreased icGSH.

To characterize the cytokine expression profile by the OMP in diabetic mice, we assessed cytokines that have been found to be related to OMP or RMP, namely, TNF- α , IL-12, IL-6, and NO. As expected, levels of TNF- α , IL-12, and NO exhibited a significant reduction in diabetic mice compared with the controls, however, IL-6 remained unchanged instead of being upregulated (Figure 2). The observed reduced cytokine expression was consistent with the decreased amount of intracellular GSH in diabetic M ϕ except IL-6, which would show an elevated production in OMP according to its definition [1].

There is clear evidence that M ϕ play an essential role in the provocation of autoimmune diabetes. In order to determine whether the OMP in diabetic mice are associated with aberrant M ϕ function, we examined the phagocytic ability of the peritoneal M ϕ using the neutral fuchsin uptake method. It was found that STZ treatment suppressed the phagocytosis activity of the peritoneal M ϕ markedly (Figure 3), though there was no marked difference between the early and the advanced phases of diabetes.

Besides the alteration in immune mediator release, we also observed evident morphological changes in cultured M ϕ s. During culture (within 7 days), normal M ϕ increased in size and differentiated from a homogeneous round shape into a morphologically heterogeneous population of fusiform- and epithelioid-shaped M ϕ . But in diabetic mice, the majority of M ϕ remained undifferentiated (data not shown).

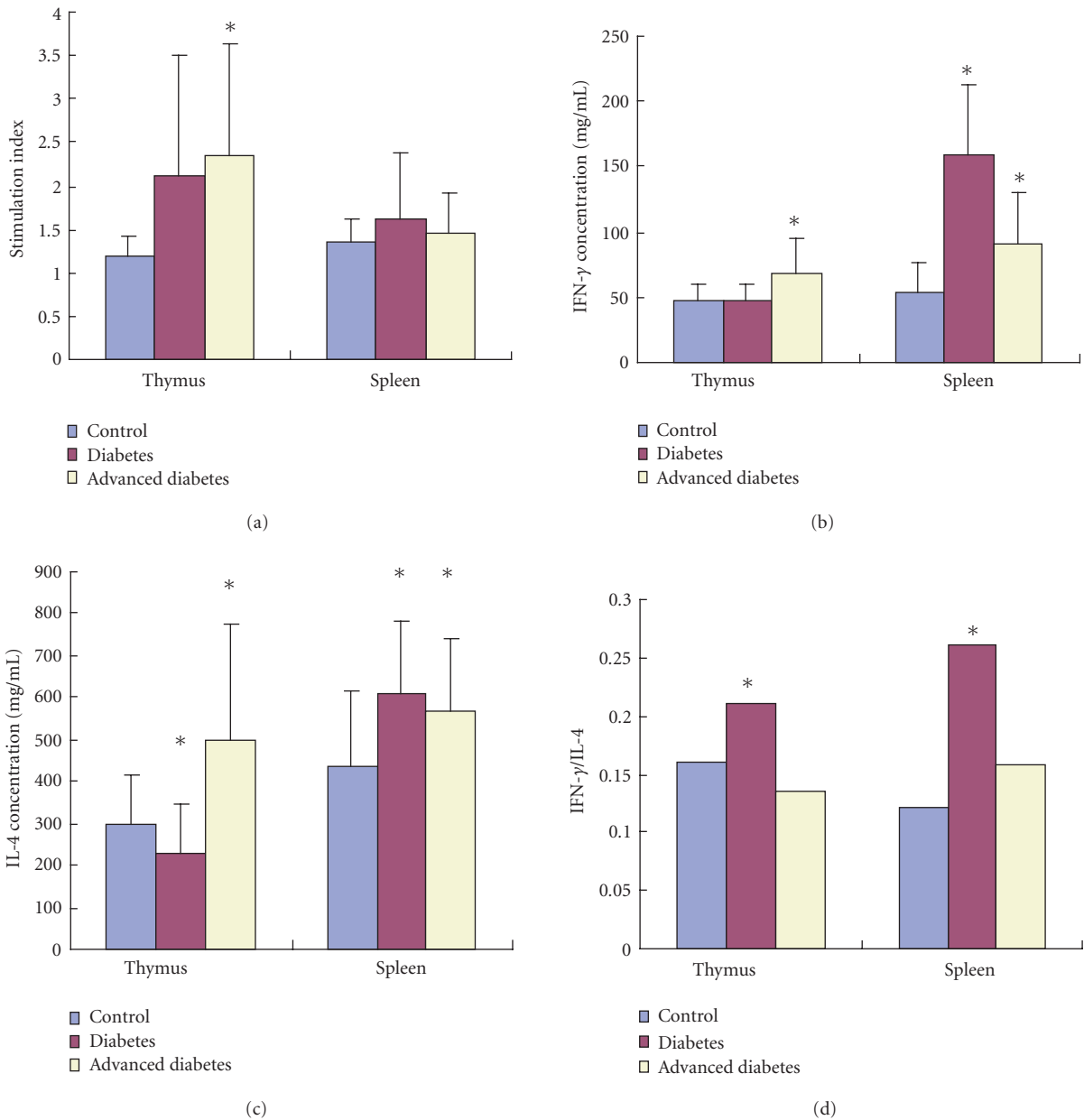


FIGURE 3. Spleen cell and thymus cell proliferation and cytokine secretion. (a) Spleen cell and thymus cell proliferation (assessed at 24 hours) in response to mitogen stimulation was affected by STZ treatment, and the stimulation indexes in the diabetic groups are all higher than the control, though not always significant. (b) Thymocytes or (c) splenocytes were stimulated with ConA (30 μ g/mL) for 48 hours before collection of conditioned medium. IL-4 and IFN- γ concentrations following stimulation were determined by ELISA. (d) The periodic change of T_H1/T_H2 balance during disease progression.

The balance of T_H1/T_H2 responses has been recognized as a critical factor in the development of type 1 diabetes, and it is well established that diabetes is associated with the development of a pathogenic T_H1 response [10]. However, it remains to be determined whether the T_H1 response varies during disease progression, and whether the variation is concomitant with the change of Mp phenotype in (multiple low-dose streptozotocin) MLD-STZ-

induced diabetes model. In the succeeding experiments, the T_H1/T_H2 balance of T cells in the spleen and thymus was analyzed. The unfractionated splenocytes or thymocytes were in vitro stimulated with ConA, and the proliferative response of cells and the cytokine levels were measured. After stimulation, the proliferative response of splenocytes or thymocytes was found to be higher in some sort than that of control mice (Figure 4a), accompanied by

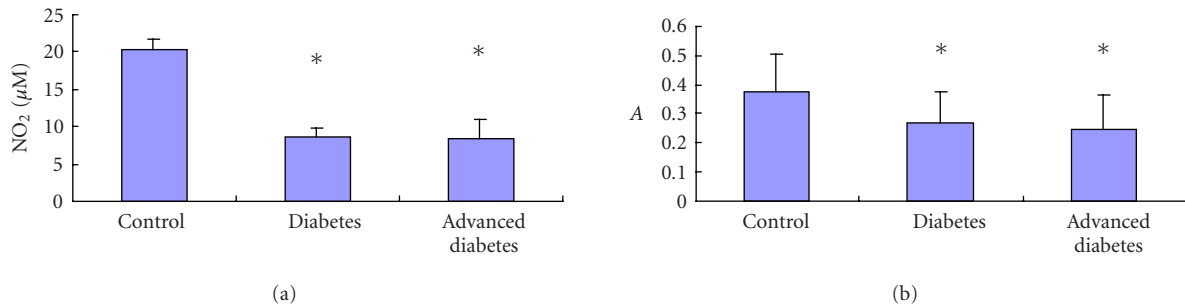


FIGURE 4. Defective phagocytic activity in diabetic mice is shown. Peritoneal Mp were harvested from the abdominal cavities of mice injected 72 hours earlier with liquid paraffin, the cells were adjusted to 5×10^6 cells/mL per well, and the ability of uptaking neutral fuchsin is indicated by A value at 570 nm.

secreting much more cytokines of IL-4 and IFN- γ (Figure 4b, 3c). Though it appeared rather complicated in the cytokines change, the ratio of IFN- γ to IL-4 showed a similar tendency in the thymus or in the spleen, with the significantly high IFN- γ /IL-4 ratio (T_H1 type phenotype) declining gradually during the disease progression (Figure 3d).

DISCUSSION

In line with the increasing evidence claiming the central role of innate over adaptive immune responses, a paradigm has been proposed on the presence of functional heterogeneity of Mp. Murata et al have divided Mp at least into two activated states based on their iGSH [1], and the role of intracellular redox status of Mp in regulating T_H1/T_H2 balance has been discussed widely [2, 3, 4, 5, 6]. However, to the author's knowledge, there is little information available in literature about the redox status of Mp in diabetic mice induced by MLD-STZ. In the present study, we conducted this study to determine whether the redox status of Mp and their cytokine production change during the development of diabetes in BALB/c mice and, additionally, if the change of the redox status of Mp is concomitant with the change of T_H1/T_H2 skewing in the spleen or the thymus. The results indicated that the peritoneal Mp were consistently skewed to Omp dominance during the disease progression, tending to produce declined cytokine expression of TNF- α , IL-12, and NO compared to those from nondiabetes controls. However, the diabetic Omp phenotype showed its abnormalities somehow characterized by its unchanged IL-6 levels and significantly reduced phagocytic activity compared with the defined Omp. On the other hand, the thymus cells and spleen cells exhibited a different T_H1/T_H2 balance shift, with a T_H1 type on the onset of diabetes, converting to T_H2 phenotypes several weeks later after diabetes occurred. The data reported here might imply that multifactor dysfunction, rather than aberrant Omp alone, may cooperate to precipitate T_H1/T_H2 imbalance of diabetic mice induced by MLD-STZ.

Streptozotocin (STZ), a potential source of oxidative stress, can penetrate into the organism generating NO and thus inducing genotoxicity [11]. It has appeared that STZ treatment generally induces an oxidative predominance in murine tissues, including liver, kidney, heart, lungs, spleen, brain, muscles, and pancreas, with decreased GSH values and increased lipid peroxidation [12]. We examined the redox status of the peritoneal Mp in MLD-STZ diabetes model and gained the similar results that Omp phenotype was dominant during the development of diabetes, consistent with the progressive decrease in GSH concentration of the serum (data not shown). This phenomenon is compatible with the findings of Carmen that a significant decline is observed in blood GSH content at the recent onset of type 1 diabetes patients, and progressive GSH depletion during diabetes evolution [13]. It is noteworthy that Omp induced by STZ treatment is not completely in accordance with the defined Omp in consideration of the rather unaltered IL-6 production. We presumed that Mp from diabetic mice might be damaged resulting from the toxic STZ, thus leading to an aberrant or "incomplete" Omp accompanied by the lower GSH content. Though the pathogenic mechanism of diabetic Omp remains elusive, one possibility is the forced infiltration of Mp into pancreas or other issues by modulating the chemotaxis of Mp. The redox status of Mp is suggested to be a crucial determinant in the regulation of the chemokine system. Saccani et al have described that H₂O₂ and the GSH-depleting drug, buthionine sulfoximine, can increase CCR2, CCR5, and CXCR4 mRNA expression to different extents and the cell migration (3-fold) in response to macrophage inflammatory protein-1 β in human monocytes [14]. Similar to it, a certain subset of Mp selectively produces C-C chemokines [15] and the redox status affects the expression of adhesion molecules critical for trans-endothelial migration of the inflammatory cell [16].

There is a growing body of evidence that aberrant cytokine production in Mp is part of a complex pathway mediating autoimmune diabetes. However, previous studies exploring inflammatory cytokine production by

Mp have not always reported consistent results [10, 17, 18]. Our data showed the generally reduced cytokine production of TNF- α , IL-12, and NO from diabetic Omp upon *in vitro* LPS (lipopolysaccharide) stimulation. However, in consideration of the increasing peritoneal Mp count in the diabetic mice, the total amount of each inflammatory mediator above is significantly higher than that of the control mice (data not shown). As larger amount of immune cells, including Mp, is also found in the islets of Langerhans in autoimmune diabetes, it is necessary to be determined whether the deficient cytokine expression itself or its relatively excessive production is relevant to the pathogenesis in diabetic Mp.

Deficiencies in phagocytosis have been associated with and may participate in the pathogenesis of both systemic and organ-specific autoimmune diseases [19]. In our animal model, we also found a significant decline in the phagocytosis activity of Mp, a possible outcome resulting from the oxidative status in diabetic Mp [20]. Yamada et al assessed the relationship between the proportion of oxidative peritoneal exudate cells (PEC) and the β -cell destruction, and suggested the proportion of the oxidative PEC as a novel marker of disease activity [21]. As clearance of dying cells is critical to the control of inflammation, engulfment of cells dying by apoptosis must occur prior to cell lysis to prevent the release of intracellular contents and possibly the generation of new antigens. So a defect in the phagocytosis may be contributory to the initiation of autoimmunity.

A number of studies have correlated diabetes with T_H1 phenotype development in the MLD-STZ diabetic animal model [8, 22, 23, 24, 25]. However, similar to other models of type 1 diabetes, some reports argued against this oversimplification. Muller et al demonstrated that MLD-STZ stimulated the production of IL-4 and IL-10, but significantly reduced IFN- γ and TNF- α levels in islets of BALB/c mice of both genders [26]. Similarly, Sitasawad indicated that both IFN- γ and IL-4 mRNA expression increased in the MLD-STZ diabetic pancreas [27]. Ins-IFN- γ transgenic mice showed apparent resistance to the induction of severe diabetes after STZ treatment compared to the control BALB/c mice [28]. In our study, the Th polarization in the spleen or the thymus varied during disease progression, with T_H1 phenotype dominant on the onset of disease converting to T_H2 phenotype several weeks after diabetes occurred. The factors dictating polarization to T_H1 phenotypes at the early stage of diabetes remain elusive. Also, it is uncertain whether the Omp participate in the conversion of T_H1 to T_H2 along with disease progression. Previous study in NOD mice showed a similar T_H1/T_H2 conversion while with a consistent sequential change of Rmp/Omp skewing [2]. In contrast to the change style, another report suggested a converse T_H1/T_H2 skewing in NOD or BB model, as T_H2-mediated attack is responsible for the early phase of IDDM, while T_H1-driven responses are responsible for the persistent and sustained attacks [29]. These findings together may

suggest that the T_H1/T_H2 shift contributes to a pathological process, which may be dictated by multiple factors, which would include the factors precipitating the disease, the local APC (antigen-presenting cell) function, and the genetic background of a given strain.

Besides the controversial T_H1/T_H2 polarization, there have been also conflicting reports regarding lymphocyte proliferative responses to mitogen either in NOD mice or in MLD-STZ-induced diabetic mice [9, 30, 31]. Here we showed a higher proliferative response of T cells (unfractionated splenocytes or thymocytes) in the diabetic mice, though not always significant, compared to the controls, in accordance with the exuberant cytokine production. One possibility is the toxic effect of STZ treatment on the subset of lymphocytes. Further experiments should be conducted to examine the expression of cell surface markers, especially the regulatory T-cell (Treg) marker of CD4⁺CD25⁺, for a loss in the number of Treg may contribute to the highly activated proliferative response. It is also under discussion whether the defective function in Mp or other APC might lead to the defective stimulation of Treg cells, thus affecting the proliferative response of lymphocytes [32]. Interestingly, a recent report suggested that the oxidation of the cellular thiol pool in T cells may play a role as an amplifying mechanism for TCR/CD3 signals in immune response [33]. It may thus provide a new insight into the abnormal lymphocyte functions under the oxidative stress in MLD-STZ-induced diabetes. Therefore, to clarify this point, experiments exploring the alteration of lymphocyte function with altered icGSH in lymphocytes of the STZ-treated mice should be carried out in the future.

In conclusion, our results presented the progressive Omp phenotype in the STZ-induced diabetic mice, and the change of T_H1/T_H2 skewing during the disease progression in the thymus and spleen. The data here implied that Omp may be relevant in the development of type 1 diabetes, however, it is not likely the only factor regulating the T_H1/T_H2 balance in MLD-STZ-induced diabetic mice. Previous studies regarding the pathogenesis of oxidative stress mainly focus on the β -cell destruction by reactive oxygen species (ROS). However, our study showed that the oxidative stress induced by STZ treatment could convert the peritoneal Mp to Omp phenotype with a generalized decrease in cytokine production and defective function. In addition, significant difference was also observed in either the blastogenesis of the splenocytes or the cytokine expression profile in the thymus and the spleen during the development of diabetes. This implies that the STZ-treatment damage in the immune cells besides Mp mediated by oxidative stress might also involve in the pathogenesis of type 1 diabetes. In future studies it is needed to be elucidated that to which extent the abnormal immune cells contribute to the pathogenesis and whether the redox regulation of the immune cells could prevent the incidence of type 1 diabetes.

ACKNOWLEDGMENTS

The authors thank L. Wang, F M. Zhang, X. Wang, and J Z. Li (Department of Cell Biology, College of Life Science, Shandong Normal University) for technical help, and Dr Yuan for continuous encouragement and helpful advice.

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