Phagocytosis of Apoptotic Cells by Macrophages From NOD Mice Is Reduced

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Macrophages limit inflammatory responses by clearing apoptotic cells. Deficiencies in apoptotic cell phagocytosis have been linked to autoimmunity. In this study, we determined the efficiency with which macrophages from diabetes-prone NOD and diabetes-resistant NOR, *Idd*5, Balb/c, and C57BL/6 mice phagocytose apoptotic thymocytes and NIT-1 insulinoma cells. Peritoneal and bone marrow–derived macrophages from NOD mice engulfed fewer apoptotic thymocytes than macrophages from Balb/c mice ($P < 0.05$). Peritoneal macrophages from NOR and *Idd5* NOD congenic mice were more proficient at engulfment than their NOD counterparts. Annexin V blockade diminished apoptotic thymocyte clearance and heat-labile serum factors augmented clearance. Binding of apoptotic thymocytes to NOD macrophages was also reduced, suggesting that the deficiency in phagocytosis may be partly attributable to a recognition defect. Peritoneal macrophages from female Balb/c and NOD mice were equally efficient in the engulfment of microspheres, suggesting that the phagocytic deficiency observed in NOD mice was specific for apoptotic cells. In summary, we have demonstrated a deficiency in phagocytic function of macrophages from NOD mice. Normal and diabetes-prone neonatal rodents have a wave of β-cell apoptosis coincident with the onset of target organ inflammation. A constitutive defect in the clearance of apoptotic β-cells may be contributory to the initiation of autoimmunity. *Diabetes* 51:2481–2488, 2002

Type 1 diabetes is an organ-specific autoimmune disease characterized by the destruction of the β-cells within the islets of Langerhans (1). The elimination of β-cells is caused by self-reactive T-cells that infiltrate the pancreatic islets (insulitis) (2). Controversy exists as to the events that initiate the activation of the islet-reactive T-cells (3). However, many studies have established that apoptosis is the major mechanism by which β-cells are destroyed (4–6).

Cell death by apoptosis triggers specific surface changes that mark the dying cell for rapid uptake and disposal, thereby preventing the release of potentially toxic and immunogenic intracellular components into tissues (7,8). Macrophages are the primary phagocytes responsible for clearance of apoptotic cells in most organs (9). Consistent with this role, they have been aptly referred to as "professional phagocytes" (9). The uptake and ingestion of apoptotic cells by semiprofessional and amateur phagocytes in the absence of functional macrophages emphasize the importance of rapidly clearing dying cells (10,11). The processes of apoptosis and phagocytosis work in concert to perform central roles in processes such as embryogenesis; mature tissue homeostasis; elimination of infected, aged, and injured cells; cellular immunity; and resolution of inflammation (12–15).

Deficiencies in apoptotic cell clearance have been associated with and may participate in the pathogenesis of both systemic and organ-specific autoimmune diseases (16). During apoptosis potential autoantigens are relocated and altered and may become the target of immune recognition, especially if the dying cells are not rapidly engulfed and destroyed by macrophages (17).

Macrophages play a pivotal role in the pathogenesis of type 1 diabetes and indirect evidence suggests that they are the earliest immune cells to invade the islets, preceding T-cell insulitis in the NOD mouse (18). Defects observed in macrophages from NOD mice include decreased MHC class I expression (19), aberrant cytokine secretion (20), and inefficient processing and presentation of antigenic material (21). NOD macrophages have also been shown to produce unusually high levels of prostoglandin-E2, which leads to the inhibition of both macrophage and dendritic cell (DC) function (22).

The number of apoptotic β-cells is significantly higher in diabetes-prone (dp) as compared with diabetes-resistant (dr) BioBreeding (BB) rats from birth until 20 days of age (23). Use of a model-based approach revealed no difference in the rate of β-cell apoptosis between dp and dr neonates. There is a marked deficiency in the ability of macrophages from BBdp neonates to engulf apoptotic cells as compared with their dr counterparts. Taken together, these findings led us to hypothesize that reduced clearance of apoptotic β-cells in neonatal dp rodents may play an important role in the initiation of β-cell autoimmunity. Similarly, there is an increased incidence of apoptotic β-cells in the pancreas of neonatal NOD mice compared with Balb/c mice (24). Whether apoptotic β-cells in NOD neonates accumulate through an increased rate of apoptosis, a decreased rate of clearance, or both is unknown. The aim of this study was to further investigate a possible role

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BMDM, bone marrow–derived macrophage; DC, dendritic cell; dp, diabetes prone; dr, diabetes resistant; H&E, hematoxylin and eosin; PS, phosphatidylserine.

for defective phagocytosis in the pathogenesis of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Animals. NOR mice (25) were purchased from The Jackson Laboratories (Bar Harbor, ME). Balb/c, C57BL/6, NOD/Lt, and *Idd5* NOD congenic (26) mice were purchased from Taconic (Germantown, NY). Experimental manipulations of the animals were performed in accordance with the regulations of the Canadian Council on Animal Care and were approved by the Animal Care Committee at Simon Fraser University.

Isolation and culture of macrophages. Macrophages were obtained by peritoneal lavage with cold complete medium (RPMI 1640; 100 units/ml penicillin/streptomycin, 10% vol/vol heat-inactivated FCS) (Life Technologies). Peritoneal cells were seeded at 4×10^5 cells/well into eight-well chamber slides (Nalge Nunc, IL) and incubated for 2 h.

Bone marrow cells were flushed aseptically from the dissected femurs of mice with sterile PBS to yield a single cell suspension. The cells were cultured in Eagle's Minimum Essential Medium (Gibco Life Technologies, NY) containing 2 mmol/l L-glutamine, 100 units/ml penicillin/streptomycin, 15 mmol/l HEPES, 10% vol/vol heat-inactivated FCS, and 100 units/ml macrophage colony-stimulating factor (Sigma, St. Louis, MO). After 4 days in culture, bone marrow–derived macrophages (BMDMs) were seeded into chamber slides at a concentration of 1×10^5 cells/well and incubated for 3 days.

Induction of apoptosis. Mouse thymocytes were irradiated with 254 nm ultraviolet C for 20 min and then cultured in serum-free complete medium for 2 h. This procedure yielded 44 ± 7% Annexin V^+/propidium iodide $(PI)^-$ thymocytes.

NIT-1 insulinoma cells (27) were maintained in F-12 medium (American *Type Cell* Culture) supplemented with 2 mmol/l L-glutamine, 1.5 g/l sodium bicarbonate, 10% vol/vol FCS, and 100 units/ml penicillin/streptomycin. Irradiation of NIT-1 insulinoma cells for 30 min followed by 3 h culture in serum-free F-12 medium resulted in 52 ± 5% Annexin V^+ cells.

Phagocytosis assays. Apoptotic cells were cocultured with macrophages at a ratio of 5:1 Annexin V^+ cells–to–macrophages for 60 min. Cells were washed, fixed, and stained with hematoxylin and eosin (H&E) (Sigma). Phagocytosis was evaluated by counting 1,000 macrophages/well. Results were expressed as either the percent phagocytosis (number of macrophages containing at least 1 apoptotic cell per 100 macrophages) or as the phagocytosis index (number of ingested apoptotic cells per 100 macrophages).

To investigate the influence of serum components on the engulfment of apoptotic thymocytes by peritoneal macrophages, phagocytosis assays were performed in the presence of normal non–heat-inactivated FCS, heat-inactivated FCS, or without serum. We also determined the ability of sera from NOD and Balb/c mice to alter the phagocytic ability of peritoneal macrophages.

For phagocytosis of inert particles, peritoneal macrophages were coincubated with a fivefold excess of 5.5 μm polystyrene microspheres (Bangs Laboratories).

To investigate the effect of annexin V blocking of phagocytosis, apoptotic thymocytes were incubated with 100 nmol/l unlabeled annexin V (BD Biosciences) for 20 min, before being coincubated with peritoneal macrophages.

To opsonize apoptotic thymocytes, they were incubated with anti-CD3 antibody (IgG2b, clone 17A2, 2 μg per 10^6 cells; Pharmingen). Target cell coating and saturation by antibody was confirmed by fluorescence microscopy after a sample of the anti-CD3–treated apoptotic thymocytes was incubated with Texas red–conjugated anti-IgG secondary antibody (1 μg per 10^6 cells; Pharmingen).

In vitro binding assays were performed at 4°C to prevent actin-based cytoskeleton rearrangements and active phagocytosis. After coincubation of peritoneal macrophages with apoptotic thymocytes, cells were gently washed three times with PBS, and conjugate numbers were counted.

To rechallenge peritoneal macrophages with apoptotic thymocytes, phagocytosis assays were performed, and after 60 min, macrophages were washed three times with PBS. Complete medium was then added to each well and macrophages were cultured for 24 h before rechallenge with fresh apoptotic thymocytes.

Immunohistochemistry. Pancreata were removed from 2-week-old mice immediately after killing, fixed, and embedded in paraffin using standard histological techniques. Tissue sections were then used in a TUNEL (TdT-mediated dUTP nick-end labeling) assay, and apoptosis was then quantified as described (4). Approximately 3,000 β-cells were counted for each animal (n = 10 animals for each strain).

Statistical analysis. The results for phagocytosis by peritoneal macrophages were analyzed using a two-way ANOVA by age and strain or treatment and strain. All other results were analyzed using two-tailed t tests.

RESULTS

Phagocytosis of apoptotic cells. Thymocytes are a convenient source of cells that can be used in apoptotic cell clearance assays (28). We used such an in vitro assay to screen for defects in apoptotic cell clearance in macrophages derived from diabetes-prone and control animals (Fig. 1*A* and *B*). At all ages macrophages from female NOD mice showed decreases in phagocytic ability relative to Balb/c macrophages (Table 1). While there was some variability in the efficiency of apoptotic cell phagocytosis between various strains, sexes, and ages, this variability was minor when compared with the differences noted between the genetically related strains, Balb/c and NOD.

The NOR mouse is a NOD-related syngenic recombinant strain that possesses ~12% C57BL/KsJ-derived genes, resulting in resistance to invasive insulitis, β-cell destruction, and diabetes (25). While peritoneal macrophages from 3-week-old female NOR mice were deficient in the phagocytosis of apoptotic thymocytes compared with Balb/c macrophages, their phagocytic ability was greater than NOD macrophages. Female NOD mice that carry the B10 allele at the *Idd5* locus exhibit a reduced frequency of diabetes as compared with their noncongenic NOD counterparts (26). Interestingly, peritoneal macrophages from *Idd5* NOD congenic mice were more proficient at engulfment than their NOD counterparts.

To determine whether the phagocytic deficiency observed in NOD mice was attributable to the unique environment of the peritoneal cavity, we also derived macrophages from bone marrow and examined their phagocytic ability. As with peritoneal macrophages, the percentages of BMDMs from female NOD mice that contained apoptotic thymocytes were lower than values observed for BMDMs from female Balb/c mice ($P = 0.02$) (Fig. 2*A*). BMDMs from male NOD mice exhibited enhanced phagocytic ability as compared with BMDMs from female NOD mice, with phagocytic function reaching levels similar to those observed for female Balb/c BMDMs (63 ± 4% of male NOD macrophages contained apoptotic thymocytes; data not shown).

Although thymocytes are a convenient cell to use in phagocytosis assays, it is unknown if all cell types display identical phagocytosis signals. We therefore examined in vitro clearance of apoptotic NIT-1 insulinoma cells (27) by peritoneal macrophages. After phagocytosis assays, fewer NOD macrophages contained apoptotic NIT-1 insulinoma cells compared with macrophages from Balb/c mice ($P < 0.05$) (Fig. 2*B*). Interestingly, the incidence of apoptotic β-cells in the pancreas of neonatal mice was higher in female NOD mice as compared with all other strains studied (Table 2).

Binding of apoptotic thymocytes. In comparison to female Balb/c mice, peritoneal macrophages from female NOD mice bound fewer apoptotic thymocytes when in vitro phagocytosis assays were conducted at 4°C (Fig. 1*C* and *D*) (16 ± 2% and 8 ± 1% of macrophages from Balb/c and NOD mice, respectively, bound apoptotic thymocytes, $P = 0.02$; data not shown). The peripheral association of apoptotic targets with macrophages and the absence of any intracellular apoptotic thymocytes confirmed that binding without internalization occurred at 4°C (29). Peritoneal macrophages from NOD mice binding more than

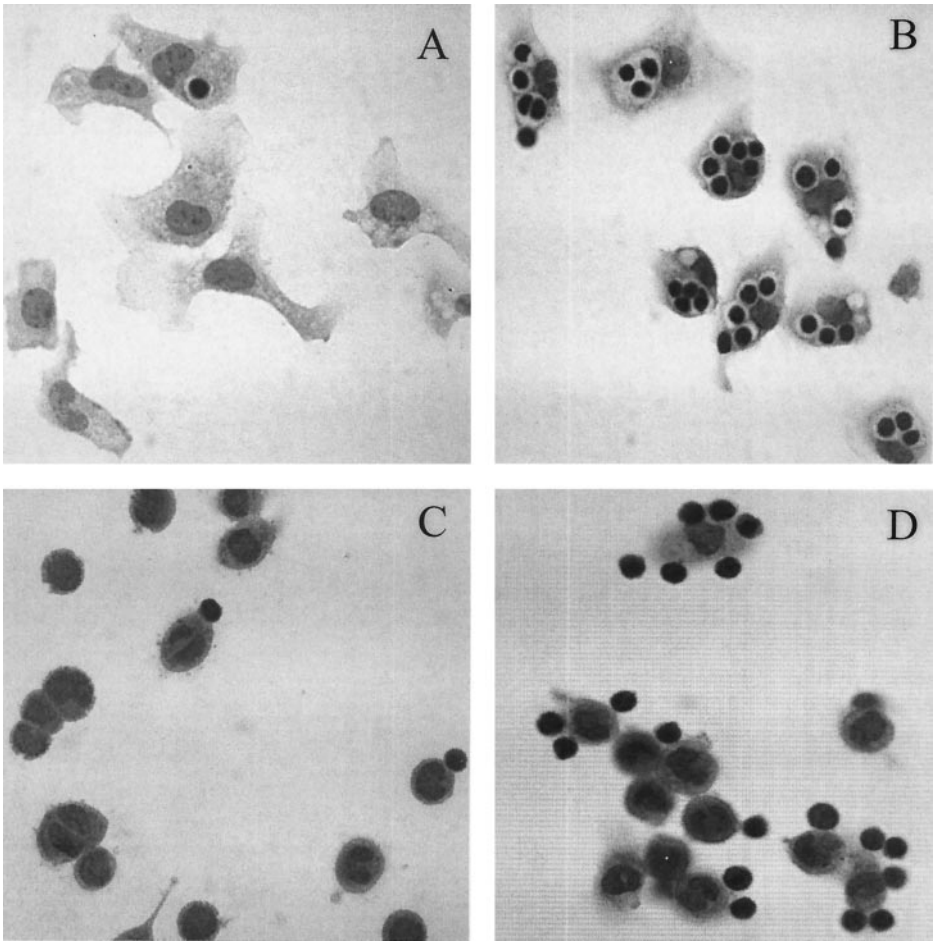

FIG. 1. Phagocytosis of autologous apoptotic thymocytes by peritoneal macrophages from a female NOD (*A*) and a female Balb/c mouse (*B*) aged 3 weeks. Photomicrographs of H&E-stained macrophages were taken 60 min after coincubation of macrophages with a fivefold excess of early apoptotic (annexin V^+) thymocytes. On microscopic examination, phagocytosed thymocytes appeared intact and displayed morphology characteristic of apoptosis. Binding without engulfment occurred when apoptotic thymocytes were coincubated with peritoneal macrophages from female NOD (*C*) and Balb/c (*D*) mice at 4°C. Original magnification ×1,000.

three apoptotic thymocytes were never observed. In contrast, some female Balb/c macrophages bound up to six apoptotic cells.

Role of heat-labile components in serum. Components present in serum have been shown to promote apoptotic cell phagocytosis by macrophages (30). Heat-labile serum factors augmented the phagocytosis of apoptotic thymocytes in all strains as the inclusion of heat-inactivated sera significantly reduced phagocytic ability (35 ± 7% and 14 ± 2% of Balb/c macrophages contained apoptotic cells in the presence of normal and heat-inactivated sera, respectively, $P < 0.05$; data not shown). The omission of sera from phagocytosis assays reduced the average number of engulfed thymocytes in all strains examined. The inclusion of heterologous sera in phagocytosis assays failed to alter phagocytic ability (data not shown).

Annexin V blockade of phagocytosis. The importance and generality of phosphatidylserine (PS) exposure on the surface of apoptotic thymocytes, as a fundamental signal for the recognition by macrophages, has been amply demonstrated (31). To determine whether PS was recognized by peritoneal macrophages from NOD and Balb/c

TABLE 1
Effect of sex and strain on the phagocytosis of autologous apoptotic thymocytes by peritoneal macrophages

Strain	Percent phagocytosis (%)				Phagocytosis index			
	1 week	2 weeks	3 weeks	12 weeks	1 week	2 weeks	3 weeks	12 weeks
Balb/c F	51 ± 2* (5)	58 ± 3* (12)	38 ± 4* (19)	45 ± 4* (10)	92 ± 5*	116 ± 9*	70 ± 13*	97 ± 15*
C57B1/6 F	28 ± 1* (8)	31 ± 3* (11)	31 ± 3* (16)	32 ± 2 (10)	38 ± 1*	52 ± 7*	46 ± 5*	55 ± 5
NOD F	12 ± 3 (6)	20 ± 2 (15)	16 ± 3 (16)	28 ± 4 (10)	18 ± 5	34 ± 3	25 ± 6	45 ± 7
NOD M	20 ± 2* (7)	25 ± 2* (17)	14 ± 1 (18)	18 ± 2* (7)	30 ± 5*	38 ± 3	18 ± 2	24 ± 4*
NOR F	—	23 ± 1 (13)	23 ± 1* (20)	42 ± 3* (10)	—	33 ± 1	35 ± 2	72 ± 6*
Idd5 F	—	27 ± 2* (9)	41 ± 2* (15)	—	—	39 ± 4	72 ± 5*	—
Idd5 M	—	28 ± 3* (5)	—	—	—	42 ± 5	—	—

Data are means ± SE. *$P < 0.05$ vs. NOD females (two-way ANOVA).

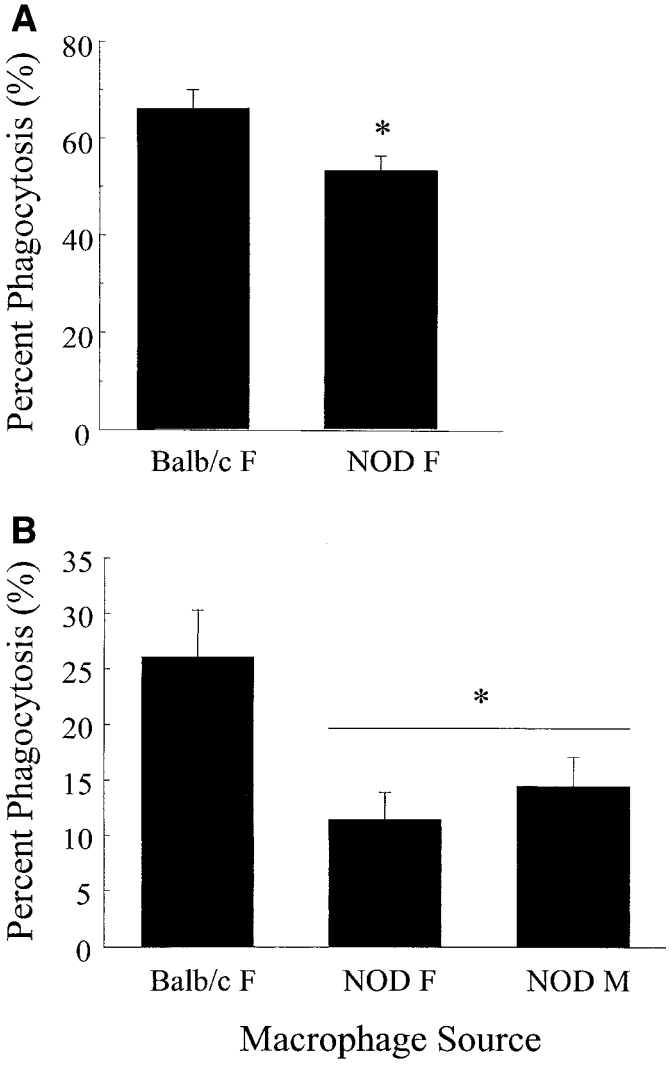

FIG. 2. ***A*****: Percent phagocytosis of apoptotic thymocytes by BMDMs from 3-week-old female Balb/c and female NOD mice. Values presented are means ± SE of independent experiments (n = 10 per strain). *P < 0.05 between strains.** ***B*****: Percentage of resident peritoneal macrophages isolated from 3-week-old female Balb/c, female NOD, and male NOD mice that contained apoptotic NIT-1 insulinoma cells after in vitro phagocytosis assays were conducted. A fivefold excess of apoptotic NIT-1 insulinoma cells was coincubated with macrophages for 60 min and macrophages were subsequently examined for the inclusion of targets. Data presented are means ± SE (n = 10 per strain). *P < 0.05 between strains.**

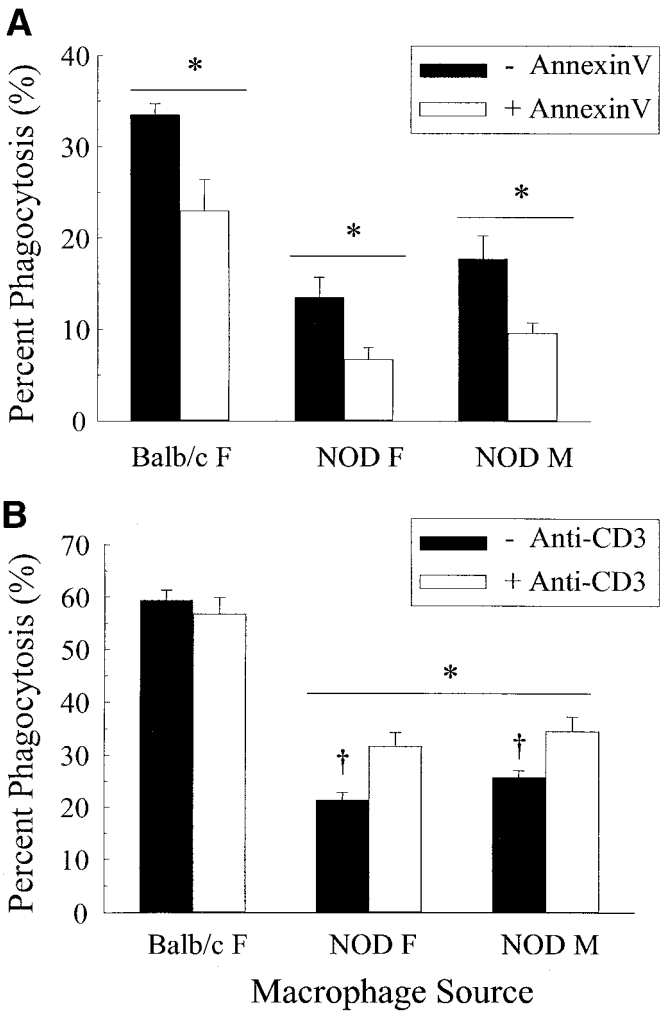

FIG. 3. ***A*****: Pretreatment of early apoptotic thymocytes with saturating amounts of annexin V decreases the percent phagocytosis by resident peritoneal macrophages from NOD and Balb/c mice aged 3 weeks. Apoptotic thymocytes were incubated with saturating amounts of annexin V, cocultured with macrophages, and the numbers of macrophages containing at least one apoptotic thymocyte after 60 min were counted.** ***B*****: Opsonization of apoptotic thymocytes with anti-CD3 antibody enhances phagocytic ability of NOD peritoneal macrophages. Data are means ± SE (n = 6 per group). *P < 0.05 compared with female Balb/c macrophages; †P < 0.05 between treatments.**

mice, we used saturating amounts of annexin V to mask PS exposed on the surface of dying thymocytes. Annexin V labeling of apoptotic thymocytes before coincubation with macrophages reduced the number of macrophages that were able to engulf dying cells (Fig. 3*A*).

TABLE 2
Fraction of apoptotic β-cells present in islets of neonatal mice of various strains

Strain	Number of apoptotic β-cells (%)
Balb/c F	0.21 ± 0.03*
C57BL/6 F	0.22 ± 0.02*
NOR F	0.29 ± 0.04*
Idd5 F	0.31 ± 0.03*
NOD M	0.33 ± 0.03*
NOD F	0.57 ± 0.02

Data are means ± SE. P < 0.05 vs. NOD females (two-tailed t test).

Opsonization of apoptotic thymocytes. To determine whether the defect in phagocytosis extended to Fc-receptor–mediated uptake, apoptotic thymocytes were opsonized with anti-CD3 antibodies and phagocytosis assays were performed. Opsonization enhanced the uptake of apoptotic thymocytes by both male and female NOD macrophages but not by macrophages from female Balb/c mice (Fig. 3*B*). Despite the enhanced phagocytosis of opsonized cells, macrophages from NOD mice were always less proficient in engulfment than Balb/c macrophages.

Phagocytosis of microspheres. To determine whether NOD macrophages had a general defect in phagocytosis, we tested macrophages for their ability to ingest microspheres. In contrast to the impaired phagocytosis of apoptotic thymocytes, macrophages from female NOD

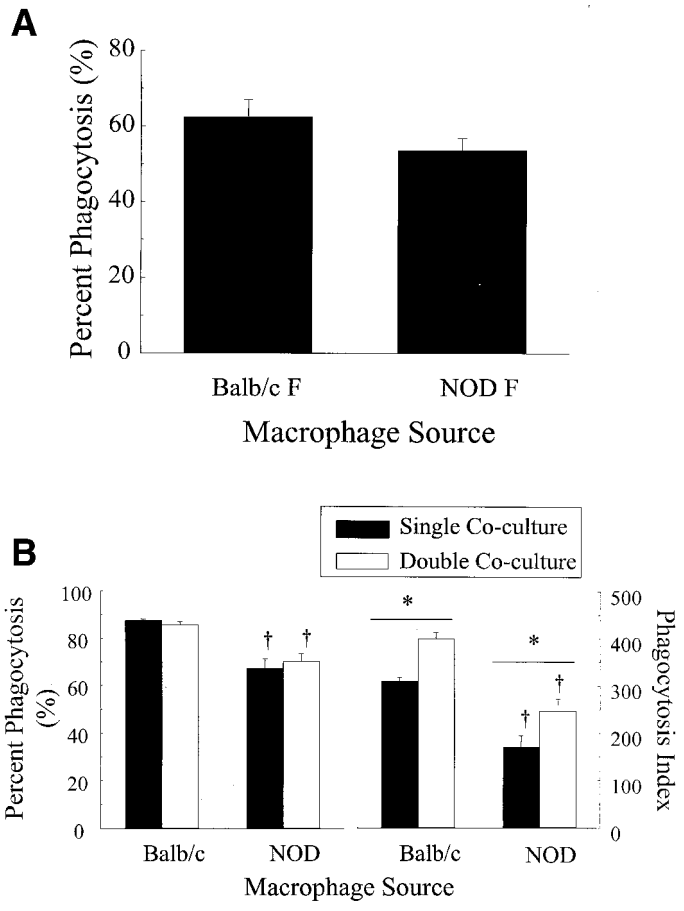

FIG. 4. ***A*: Percentage of resident peritoneal macrophages from 3-week-old female NOD and female Balb/c mice that contained 5.5-μm microspheres after in vitro phagocytosis assays were performed. Values presented are means ± SE (n = 10 per strain).** ***B*: Effects of single and double coculture of apoptotic thymocytes with peritoneal macrophages on percent phagocytosis (left panel) and phagocytosis index (right panel). Resident peritoneal macrophages were incubated 2 h before an initial 60-min interaction with either apoptotic thymocytes or medium alone. Macrophages were then washed and cultured in complete medium for a further 24 h before washing and interaction with fresh apoptotic thymocytes. The number of engulfed apoptotic thymocytes still observable before rechallenge with a second meal of apoptotic cells was subtracted from the number of fresh apoptotic thymocytes engulfed to determine phagocytosis values after double culture with targets. Data are means ± SE, n = 8 per group. *P < 0.05 between single and double coculture with apoptotic thymocytes; †P < 0.05 between strains after single or double coculture.**

mice were fully competent in the phagocytosis of polystyrene beads (Fig. 4*A*).

Rechallenge of macrophages with apoptotic thymocytes. Previous uptake of apoptotic cells can modulate the ability of macrophages to subsequently ingest apoptotic cells (32,33). Resident peritoneal macrophages may engulf apoptotic cells in vivo before collection for analysis, and this could affect their subsequent function. We wanted to determine whether macrophages from female NOD mice were more susceptible to such modulation. The numbers of ingested apoptotic cells per 100 macrophages for both NOD and Balb/c macrophages were increased after macrophages were rechallenged with a second apoptotic meal (Fig. 4*B*). Despite the ability of NOD macrophages to phagocytose increased numbers of apoptotic cells after repeated feeding, their phagocytic ability remained significantly reduced as compared with Balb/c macrophages (Fig. 4*B*).

DISCUSSION

This study is the first to report a defect in the phagocytosis of both apoptotic thymocytes and transformed β-cells (NIT-1 insulinoma cells) by peritoneal and BMDMs from mice predisposed to the development of type 1 diabetes.

Peritoneal macrophages from female NOD mice of all ages consistently exhibited reduced phagocytic ability compared with the genetically related Balb/c strain. However, there was some variability in the efficiency of apoptotic cell phagocytosis between various strains, sexes, and ages. The observation that macrophages from the genetically unrelated and diabetes-resistant C57BL/6 strain performed inferior to Balb/c macrophages may be partly attributable to H-2 genes controlling phagocytic function of murine macrophages. Peritoneal macrophage function, in both autoimmune-prone and autoimmune-resistant mouse strains, has been reported to increase with age (34). Consistent with this, we observed enhanced phagocytic function in older mice. The observation that peritoneal macrophages from 12-week-old female NOD mice displayed a greater phagocytic efficiency than age-matched male mice is consistent with reported sex differences in macrophage function, indicating that the ingestion capacity of macrophages is higher in adult female mice (35,36). Nonetheless, the strain-, sex-, and age-based variability was minor when compared with the differences noted between peritoneal macrophages from Balb/c and NOD mice.

Since peritoneal macrophages are derived from bone marrow precursors, we wanted to determine whether the defect seen in NOD resident peritoneal macrophages is acquired after the precursors leave the bone marrow (either due to a developmental stage of the macrophage or due to environmental influences) or if it is cell autonomous. After the in vitro derivation of macrophages from bone marrow precursors, the phagocytic ability of the female NOD phenotype was still reduced as compared with Balb/c mice. Our results thus suggest that the phagocytic defect in female NOD mice is attributable, in part, to intrinsic factors. We cannot exclude a possible contributory role for environmental factors in this engulfment deficiency, because peritoneal macrophages generally exhibited reduced phagocytic ability as compared with BMDMs. Indeed, phenotypic differences between BMDMs and resident peritoneal macrophages from other mouse strains have been previously reported (37). Male peritoneal NOD macrophages from young mice were slightly more efficient in phagocytosis than female macrophages but still less efficient than macrophages from Balb/c mice. Likewise, BMDMs from male NOD mice performed slightly better than those from female NOD mice, with the former displaying similar phagocytic ability to female Balb/c BMDMs. These sex differences were minor when compared with interstrain differences and suggest that other factors may contribute to the differential sex susceptibility to diabetes in NOD mice.

The rapid uptake of apoptotic cells before they become necrotic suggests that dying cells display specific recognition signals for phagocytes. To date, the only surface change that has been directly and consistently demonstrated to promote the phagocytosis of apoptotic thymocytes by macrophages is PS (31). To determine whether

the observed reductions in apoptotic cell uptake were related to defects in PS recognition, we blocked the exposed PS on the dying thymocytes using saturating amounts of annexin V. The presence of annexin V–labeled apoptotic thymocytes significantly reduced the number of apoptotic cells phagocytosed by macrophages from both phenotypes. Comparable reductions in both the percent phagocytosis and phagocytosis indexes were recorded when macrophages from NOD and Balb/c mice were challenged with apoptotic cells whose surface PS was blocked with annexin V. This suggests that the specific deficiency in phagocytosis observed in NOD mice is unlikely to be attributable to defects in PS recognition. Whether NOD macrophage recognition of other changes to apoptotic cells [including expression of sugars (38), loss of surface expression of some GPI-linked antigens (such as CD16) (39), or other currently uncharacterized molecules] is defective in NOD mice is unknown. Our finding that NOD macrophages bind fewer apoptotic thymocytes suggests that the deficiency in phagocytosis may be partly attributable to a recognition defect. This could be due to a decreased number of receptors, reduced turnover of receptors, or decreased avidity of receptors that play important roles in the binding and phagocytosis of apoptotic cells.

Fc-receptors on macrophages mediate the phagocytosis of antibody-containing complexes; therefore, we sought to determine whether opsonizing apoptotic thymocytes with anti-CD3 antibody could eliminate the deficiency in phagocytosis by NOD macrophages. Opsonization of apoptotic thymocytes improved uptake by NOD macrophages but not Balb/c macrophages, the latter demonstrating near optimal uptake in the absence of opsonin. Nevertheless, peritoneal macrophages from NOD mice were unable to ingest opsonized apoptotic thymocytes as efficiently as macrophages from Balb/c mice. The incomplete correction of uptake by opsonization could be due to multiple factors including deficient Fc-receptor expression, binding site competition, and downstream phagocytic defects. A general phagocytic defect is unlikely, as polystyrene bead uptake was essentially normal.

Phagocytosis of cells targeted for removal by macrophages is a multistep process initiated by recognition and binding of the target followed by its internalization. Sambrano et al. (40) propose that the PS receptor on macrophages functions primarily as an adhesion receptor for apoptotic cells, while the initiation of engulfment requires engagement of Fc-receptors to stimulate actin polymerization and internalization of the dying cell. The fact that neither PS-induced nor Fc-induced signaling normalize apoptotic cell uptake in NOD macrophages suggests that novel surface receptors or signaling molecules common to both pathways may be perturbed. In this regard, further analysis of NOD congenic mice may provide genetic clues to these pathways.

It has been reported that serum components, such as complement proteins, bind to apoptotic cells and facilitate their uptake in vitro (30,41). Deficiencies in the early complement component, C1q, predispose humans and mice to lupus-like autoimmune disease (42). C1q-deficient mice develop proliferative glomerulonephritis characterized by marked accumulation of apoptotic cells in the kidney (43,44). Macrophages from C1q-deficient mice are defective in the phagocytosis of apoptotic cells in vivo (45). In our study, the marked enhancement of phagocytosis by macrophages from both NOD and Balb/c strains induced by serum factors was abrogated by heat-inactivating sera, suggesting that complement components are responsible for the uptake of apoptotic thymocytes by macrophages from both NOD and Balb/c phenotypes. As this phenomenon was observed for mice, regardless of their disposition toward diabetes development, heat labile components in serum are unlikely responsible for the specific phagocytic defect in NOD peritoneal macrophages. Due to the short duration of the phagocytosis assay (1 h), it is improbable that macrophages and/or thymocytes may have secreted factors that either positively or negatively influenced the uptake of apoptotic thymocytes. Consistent with previous reports, we found that when in vitro phagocytosis assays were performed in medium alone, the average number of engulfed thymocytes was reduced in all strains examined (30). This suggests that both heat labile and nonlabile components of serum facilitate the uptake of apoptotic thymocytes. We next wanted to determine whether the inclusion of serum from Balb/c mice could increase the phagocytic ability of NOD peritoneal macrophages. The number of apoptotic thymocytes engulfed by both NOD and Balb/c peritoneal macrophages was not affected by the serum source present in the assay. Therefore, deficiencies in contributory serum factors were unlikely responsible for the phagocytic defect in NOD mice.

Rechallenging peritoneal macrophages from lupus-prone mice with apoptotic cells led to an increased capacity to phagocytose fresh apoptotic cells (32). Further, phagocytic activity increases after prolonged in vitro culture (33). Therefore, we sought to determine whether rechallenging peritoneal macrophages from NOD mice with apoptotic thymocytes would abrogate the engulfment deficiency. The number of macrophages from both NOD and Balb/c phenotypes that had phagocytosed apoptotic thymocytes after a second challenge was similar to when macrophages were given only a single feed of dying cells. In contrast, both NOD and Balb/c macrophages were able to increase the number of apoptotic cells engulfed (phagocytosis index) when presented with a second apoptotic meal. If this increased phagocytosis is due to direct engulfment-induced changes in the macrophage or autocrine cytokine release is still unknown. Regardless of whether a single or double coculture of apoptotic thymocytes occurred, the phagocytic ability of NOD peritoneal macrophages remained significantly less as compared with Balb/c macrophages.

Interestingly, peritoneal macrophages from both female NOR and female *Idd5* mice were more proficient at engulfment than NOD macrophages. While the NOR strain retains the majority (88%) of the NOD genotype, the former is resistant to β-cell destruction and diabetes development (25). NOR mice do exhibit peri-insulitis; however, unlike their NOD counterparts, antigen-presenting cell infiltration of islets is not accompanied by T-cell invasion and progression to invasive insulitis, suggesting that the transition from benign to destructive insulitis is prevented in this strain (46). Likewise, the *Idd5* suscepti-

bility locus affects the development of invasive insulitis in the NOD mouse. *Idd5* congenic NOD mice contain the B10 allele at the *Idd5* locus and exhibit a reduced frequency of diabetes as compared with female NOD mice (26). Additionally, NOR mice lack a significant portion of the NOD-derived *Idd5* allele. Collectively, these findings have led to the hypothesis that the *Idd5* locus in NOD mice may play a pivotal role in recruiting T-cells to islets, thereby affecting the development of destructive insulitis (46). As both NOR and *Idd5* NOD congenic macrophages have a normalization of the phagocytic deficiency observed in NOD mice, it is attractive to speculate that candidate genes within the *Idd5* locus affect macrophages phagocytic function.

In this work, we have documented a defect in macrophage apoptotic cell clearance. We have also found an increased incidence of apoptotic β-cells in neonatal female NOD islets as compared with control strains. An increased rate of β-cell apoptosis, as is noted during tissue remodeling, may overburden the NOD phagocytic system as we show that NOD macrophages have an intrinsic defect in their ability to clear apoptotic debris. In general, when macrophages are present with DCs in phagocytosis assays, the proinflammatory presentation of apoptotic material is markedly inhibited. This is largely attributable to the highly efficient phagocytosis and degradation of apoptotic cells by the macrophages, sequestering the apoptotic cells from the DCs. Furthermore, the consequence of macrophage ingestion of apoptotic cells is commonly the release of immunosuppressive cytokines such as tumor growth factor (TGF)-β (47). Rovere et al. (48) generated in vitro data suggesting that large numbers of apoptotic cells may enhance immunostimulatory DC maturation and, therefore, their ability to process and present antigens from apoptotic cells to T-cells. Failure to clear dying cells may reflect an in vivo imbalance between the number of dead cells and the local availability of functional scavenger phagocytes. Factors that increase apoptosis (such as virus infections, local inflammation, and neonatal tissue remodeling) and factors that decrease the phagocytic ability of macrophages (such as phagocytic exhaustion and cytokine milieu) may contribute to the accumulation of uncleared apoptotic material and the breaking of immune tolerance (49). Indeed, intravenous injection of nonimmune mice with syngeneic apoptotic thymocytes results in the transient production of autoantibodies, and defects in phagocytosis have been correlated to increased autoantibodies (50). Collectively, these results suggest that increased apoptosis and/or decreased phagocytic ability play a major role in the pathogenesis of autoimmune syndromes. We thus suggest that the intrinsic macrophage defect that we describe herein contributes to the autoimmune phenotype of the NOD mouse.

Undoubtedly, the prevention of autoimmunity depends on both the removal of apoptotic cells and on an active suppression of inflammatory mediator production (47). Whether the observed macrophage phagocytic defect results in deficient immunoregulatory cytokine production is currently under investigation. Individuals susceptible to type 1 diabetes development may, likewise, carry a defect in the ability to efficiently bind, engulf, and degrade apoptotic β-cells, or to mount an adequate anti-inflammatory response upon ingestion of apoptotic material. To our knowledge, this aspect of macrophage function has yet to be explored in diabetic kindreds. Multiple pharmacological agents are known to modulate macrophage function. Studies to elucidate the different mechanisms used by macrophages from susceptible and resistant individuals to recognize and phagocytose apoptotic cells and the events following the engulfment of apoptotic cells may lead to novel strategies for therapeutic intervention.

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