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Immunology

Hematopoietic stem-cell defects underlying abnormal macrophage development and maturation in NOD/Lt mice: Defective regulation of cytokine receptors and protein kinase C

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ABSTRACT **The immunopathogenesis of autoimmune insulin-dependent diabetes in NOD mice entails defects in the development of macrophages (Mφs) from hematopoietic precursors. The present study analyzes the cellular and molecular basis underlying our previous finding that the Mφ growth factor colony-stimulating factor 1 (CSF-1) promotes a reduced level of promonocyte proliferation and Mφ development from NOD bone marrow. CSF-1 stimulation of NOD marrow induced Mφs to differentiate to the point that they secreted levels of tumor necrosis factor α equivalent to that of controls. However, CSF-1 failed to prime NOD Mφs to completely differentiate in response to γ-interferon, as shown by their decreased lipopolysaccharide-stimulated interleukin 1 secretion. These defects, in turn, were associated with an inability of CSF-1 to up-regulate c-*fms* (CSF-1 receptor) and *Ifgr* (γ-interferon receptor) expression. Even though the combination of CSF-1 and γ-interferon up-regulated c-*fms* and *Ifgr* transcript levels in NOD Mφs to levels induced in control Mφs by CSF-1 alone, the protein kinase C activities coupled to these receptors remained 4-fold lower in NOD Mφs than in Mφs derived from the marrow of diabetes-resistant NON and SWR control mice. Despite expressing the diabetogenic *H-2*^{g7} haplotype, Mφs derived from cytokine-stimulated marrow of the NON.*H-2*^{g7} congenic stock were functionally more mature than similarly derived Mφs from NOD mice. Whereas diabetes resistance was abrogated in 67% of irradiated (NOD × NON)F_1 females reconstituted with NOD marrow, no recipients became diabetic after reconstitution with a 1:1 mixture of marrow from NOD and the congenic stock. Thus, failure to develop functionally mature monocytes may be of pathogenic significance in NOD mice.**

NOD is an inbred mouse strain characterized by a high incidence of T-cell-mediated autoimmune insulin-dependent diabetes (1). Genetic outcross of NOD with the closely related but diabetes-resistant NON inbred strain has shown that diabetogenesis results from complex polygenic interactions between the rare *H-2*^{g7} major histocompatibility complex (MHC) haplotype (K^d, $I\text{-}A^{g7}$, $I\text{-}E^{null}$, D^b) of NOD and non-MHC-linked susceptibility modifiers (2). These diabetogenic interactions are initiated at the hematopoietic stem-cell level since diabetes resistance in (NOD × NON)F_1 hybrids is abrogated by lethal irradiation and reconstitution with NOD bone marrow but not NON/Lt or F_1 bone marrow (3, 4). Several lines of evidence indicate that *H-2*^{g7}-expressing antigen-presenting cells (APCs), such as macrophages (Mφs) derived from NOD stem cells, fail to mediate the clonal deletion of β-cell autoreactive T cells and/or to activate immunoregulatory T cells. These include the finding that marrow-derived APCs, but not thymic epithelial cells, expressing the diabetes-resistant *H-2*^{nb1} haplotype (K^b, $I\text{-}A^{nb1}$, $I\text{-}E^k$, D^b) of NON blocked the development of diabetogenic T cells from NOD bone marrow (4). In addition, the inability of NOD APCs to activate immunoregulatory T cells in a syngeneic mixed-lymphocyte reaction was also found to be associated with homozygous expression of *H-2*^{g7} (5).

Interactions between the *H-2*^{g7} haplotype and non-MHC-linked background genes also underlie the Mφ developmental anomalies characteristic of NOD mice. NOD bone marrow cells proliferate poorly in response to several myeloid growth factors (6, 7). The myeloid growth factor, colony-stimulating factor 1 (CSF-1) generates fewer phenotypically mature (Mac-3$^+$) Mφs from NOD marrow than from diabetes-resistant strains (6). This is consistent with the finding that peritoneal Mφs from NOD mice are poor interleukin 1 (IL-1) secretors (5, 8), since Mφs reportedly do not acquire the ability to secrete IL-1β until the later stages of differentiation (9). Further evidence for dysregulated monocyte function in NOD mice is reflected by the aberrant responses of cells in this lineage to γ-interferon (IFN-γ) stimulation. As expected, IFN-γ, which induces Mφ differentiation and activation, inhibited CSF-1-stimulated proliferation of bone marrow cells from diabetes-resistant strains (6). In contrast, IFN-γ aberrantly increased CSF-1-stimulated proliferation of NOD bone marrow cells and increased the number of Mac-3$^+$ progeny generated to levels equivalent to that produced from the marrow of control strains stimulated with CSF-1 alone (6). In addition, as a result of defects in background-specific trans-regulatory factors, IFN-γ also aberrantly down-regulated *H-2*^{g7} MHC class I expression in NOD peritoneal Mφs (6). Collectively, these results indicate that non-MHC-linked genes either independently or through interaction with the diabetogenic *H-2*^{g7} haplotype contribute to impairments in monocyte differentiation and function in NOD mice. Antigens that are processed and presented in an inefficient fashion can continue to stimulate T-cell proliferative responses in the periphery but are unable to induce tolerance (10, 11). Similarly, activation-driven T-cell death requires quantitatively more antigenic stimulation than is required to trigger T-cell proliferation (12). In addition, the stimulation of immunoregulatory T cells requires a more highly activated APC than is required to trigger effector T cells (13). Thus, in synergy with the diabetogenic *H-2*^{g7} haplotype, the non-MHC-linked background genes contributing to defects in Mφ development may contribute to diabetogenesis in NOD mice by decreasing the ability of APCs to process and/or present β-cell autoantigens in a manner quantitatively sufficient to induce tolerance. Therefore, the present study was conducted to assess the functional consequences of impaired Mφ

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Abbreviations: APC, antigen-presenting cell; CSF-1, colony-stimulating factor 1; IFN-γ, γ-interferon; IL-1, interleukin 1; LPS, lipopolysaccharide; Mφ, macrophage; MHC, major histocompatibility complex; PKC, protein kinase C; TNF-α, tumor necrosis factor α.

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development in NOD mice and to analyze the mechanisms underlying these maturational defects.

MATERIALS AND METHODS

Mice. NOD/Lt and NON/Lt mice have been maintained in our research colony by brother–sister mating since being obtained from George Eisenbarth (Joslin Diabetes Center, Boston) at the 32nd and 35th generation of inbreeding, respectively. The previously described (2, 14) diabetes-resistant NOD.NON-*H-2*^{nb1} (designated NOD.*H-2*^{nb1}) and NON.NOD-*H-2*^{g7} congenic strains (designated NON.*H-2*^{g7}) were utilized at the 21st and 13th backcross generations, respectively. SWR/Bm (*H-2*^{q}) mice were kindly supplied by Wesley Beamer (The Jackson Laboratory). Nondiabetic female mice between 8 and 12 weeks of age were used for all experiments and were maintained under specific pathogen-free conditions and allowed free access to food (diet 96W, Emory Morse, Guilford, CT) and acidified drinking water.

IL-1 and Tumor Necrosis Factor α (TNF-α) Production by Bone Marrow-Derived Mφs. Bone marrow cells were suspended at 2.0×10^6 cells per ml in culture medium (5) containing recombinant human CSF-1 (Cetus) at 500 units/ml with and without recombinant rat IFN-γ at 10 units/ml (kindly supplied by P. van de Meide, Rijswijk, The Netherlands) and cultured for 4 days at 37°C in a humidified 5% CO_2/95% air atmosphere. The proliferative responses and level of Mac-3 expression of the cells emanating from marrow cultured with this source and concentration of CSF-1 were found to be equivalent to that induced by CSF-1 at 25 units/ml [obtained from Genzyme—the CSF-1 used in our previous study (6)]. After 4 days in culture, the growth factor-containing medium was replaced with either fresh medium alone or with medium containing lipopolysaccharide (LPS) at 10 μg/ml. After an additional 24-h incubation, the culture supernatants were harvested and assayed for IL-1 content in a C3H/HeJ thymocyte comitogenic assay as described (5). Data are presented as units of IL-1 (mean ± SEM) compared to a recombinant murine IL-1α standard (Hoffman–La Roche). TNF-α content of the culture supernatants was assessed as described (15) in a WEHI 164 growth inhibition assay using recombinant murine TNF-α (Genzyme) as a standard.

Analysis of CSF-1 Receptor and IFN-γ Receptor mRNA Levels. Total cellular RNA was extracted as described (16) from marrow cells cultured for the indicated periods of time in medium alone or with CSF-1 in the presence and absence of IFN-γ. Northern blot analysis was performed as described (17) using the SM3 cDNA to detect c-*fms* transcripts encoding the CSF-1 receptor (18) and the MUIFN-γR cDNA to detect *Ifgr* transcripts encoding the IFN-γ receptor (19). The blots were also hybridized with a chicken β-actin cDNA probe (kindly supplied by Hendrick Bedigian, The Jackson Laboratory) to verify that equal quantities of RNA were present in all samples.

Assay of Protein Kinase C (PKC) Activity Induced by CSF-1. Triplicate cultures of 10^5 bone marrow cells were seeded into flat-bottom 96-well microtiter plates in 0.2 ml of medium containing CSF-1 at 500 units/ml with and without IFN-γ at 10 units/ml. At the indicated times, cellular PKC activity was assessed as described (20). Briefly, the medium was removed and the cells were permeabilized with digitonin (10 μg/ml) in a reaction buffer also containing 100 μM [γ-^{32}P]ATP (Amersham, PB.170) and a 9-aa (VRKRTLRRL) synthetic substrate (Sigma, V-2131; 100 μM) corresponding to the major PKC phosphorylation site of the epidermal growth factor receptor (21). The cells were incubated for 15 min at 37°C, the reaction was terminated by addition of 25% (wt/vol) trichloroacetic acid, and 45 μl of reaction supernatant was spotted onto Whatman P81 filters. The filters were sequentially washed with 75 mM phosphoric acid and 75 mM sodium phosphate (pH 7.5), and then radioactivity was measured in a β scintillation counter (LKB) to assess PKC-driven phosphorylation of the synthetic substrate. Data are presented as cpm (mean ± SEM) corrected for nonspecific ^{32}P incorporation obtained in the absence of substrate and for the protein concentration of the reaction supernatant.

Competitive Bone Marrow Reconstitution Studies. Diabetes-resistant (NOD × NON)F_1 female mice were lethally irradiated (1000R; 1R = 0.258 mC/kg) at 4 weeks of age and reconstituted as described (4) with a 1:1 mixture of T-cell-depleted NOD and NON.*H-2*^{g7} bone marrow cells (2.5×10^6 marrow cells from each strain). Controls consisted of F_1 hybrids reconstituted with NOD bone marrow alone. Chimeras were monitored through 40 weeks of age for the development of glycosuria with Tes-Tape (kindly supplied by Eli Lilly). Upon diabetes onset or reaching 40 weeks of age, splenocytes from the chimeras were typed as described (4, 22) by two-color flow cytometric analysis with the Thy-1.2 (NOD type)-specific monoclonal antibody HO13-4.9 and the Thy-1.1 (NON.*H-2*^{g7} type)-specific monoclonal antibody HO22-1 to estimate the percentage of leukocytes derived from each marrow population.

RESULTS

Comparison of IL-1 and TNF-α Secretion by Bone Marrow-Derived Mφs from NOD Mice and Diabetes-Resistant Strains. Fig. 1 compares LPS-stimulated IL-1 secretion of Mφs derived from NOD marrow cultured in CSF-1 with and without IFN-γ to that of similarly derived Mφs from diabetes-resistant control strains. Our previous study (6) documented that fewer phenotypically mature (Mac-3$^+$) Mφs are generated from CSF-1-stimulated NOD marrow than from CSF-1-stimulated marrow of the same diabetes-resistant control strains utilized in the present study (NON, SWR, NOD.*H-2*^{nb1}, and NON.*H-2*^{g7}). Despite this difference in degree of phenotypic maturation, the levels of LPS-stimulated IL-1 secretion (7–12 units/ml) were equivalent in CSF-1-elicited Mφs from all of these strains (Fig. 1). Similarly, the level of LPS-stimulated TNF-α secretion from NOD Mφs derived in CSF-1 alone (42.0 ± 2.5 units/ml) was equivalent to that of similarly derived Mφs from SWR/Bm controls (40.0 ± 1.9 units/ml). We have demonstrated (6) that NOD marrow must be cultured in a combination CSF-1 and IFN-γ to generate as many phenotypically mature (Mac-3$^+$) Mφs as are generated from the marrow of diabetes-resistant control strains cultured in CSF-1 alone. Mφs derived by culturing marrow from diabetes-resistant NON, SWR, and NON.*H-2*^{g7} mice in a

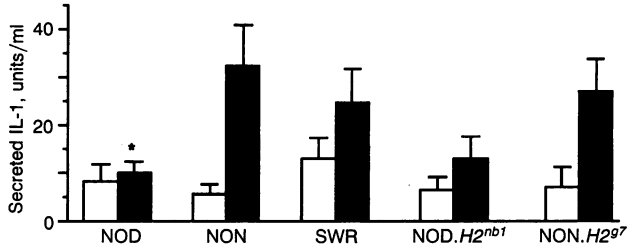

FIG. 1. Comparison of IL-1 secretion by Mφs derived from the bone marrow of NOD mice and diabetes-resistant strains cultured in CSF-1 with (solid bars) and without (open bars) IFN-γ. Mφs derived from bone marrow cells cultured for 4 days in the various cytokine combinations were stimulated for an additional 24 hr with medium containing LPS (10 μg/ml). IL-1 secretion was then assayed. Data represent the LPS-stimulated IL-1 secretion (mean ± SEM) from six experiments. *LPS-stimulated IL-1 secretion from NOD Mφs derived in a combination of CSF-1 and IFN-γ significantly less ($P < 0.05$ by Student's t test) than from similarly derived Mφs from diabetes-resistant NON, SWR, and NON.*H-2*^{g7} mice.

combination of CSF-1 and IFN-γ secreted high levels of IL-1 (25–32 units/ml) after LPS stimulation (Fig. 1). However, similarly derived Mφs from NOD mice secreted significantly less IL-1 (≈10 units/ml) after LPS stimulation (Fig. 1). After 20 backcross generations, the genetic background of the NOD.*H-2*^{nb1} congenic stock is almost completely NOD-like except for the small congenic segment encompassing the *H-2*^{nb1} MHC haplotype of NON on chromosome 17. LPS-stimulated IL-1 secretion from Mφs derived from NOD.*H-2*^{nb1} congenic marrow cultured in a combination of CSF-1 and IFN-γ (≈13 units/ml), although slightly higher, did not statistically differ from that of similarly derived Mφs from standard NOD/Lt mice. Collectively, these results clearly demonstrate that while NOD Mφs derived in a combination of CSF-1 and IFN-γ are phenotypically equivalent (as assessed by Mac-3 expression) to similarly derived Mφs from diabetes-resistant strains (6), as a result of NOD-specific background genes, these cells fail to become functionally mature as assessed by a decreased ability to secrete IL-1 after LPS stimulation. These same genetically controlled defects in myeloid development would explain the monokine secretory defect documented for peritoneal Mφs from adult NOD mice (5, 8).

Analysis of CSF-1 Receptor and IFN-γ Receptor mRNA Levels in Bone Marrow-Derived Mφs from NOD Mice and Diabetes-Resistant Strains. The receptor for CSF-1 is encoded by the c-*fms* protooncogene locus on mouse chromosome 18 (23, 24). Northern blot analysis was performed to determine whether the Mφ development and activation defects characteristic of NOD mice were associated with impaired or delayed expression of c-*fms*. Only low levels of c-*fms* transcripts were detected in freshly isolated bone marrow cells from NOD mice and diabetes-resistant NON and SWR control mice and in marrow cells from these same strains cultured for 24 hr in CSF-1 alone (data not shown). However, after 4 days in culture with CSF-1, much higher levels of c-*fms* transcripts were detected in bone marrow cells from diabetes-resistant NON and SWR control mice than in marrow cells from diabetes-susceptible NOD mice (Fig. 2). IFN-γ inhibits CSF-1-stimulated proliferation of NON and SWR marrow cells (6), and as shown in Fig. 1, primes Mφs to become functionally more competent. In contrast, IFN-γ aberrantly increases CSF-1-stimulated proliferation of NOD marrow cells (6), and the Mφs generated remain functionally immature (Fig. 1). The differential effect of IFN-γ on proliferation and activation of CSF-1-stimulated marrow from diabetes-susceptible and -resistant strains correlated with the levels of c-*fms* expression. As shown in Fig. 2, the level of c-*fms* transcripts in NOD marrow cells cultured in a combination of CSF-1 and IFN-γ was greater than that of NOD

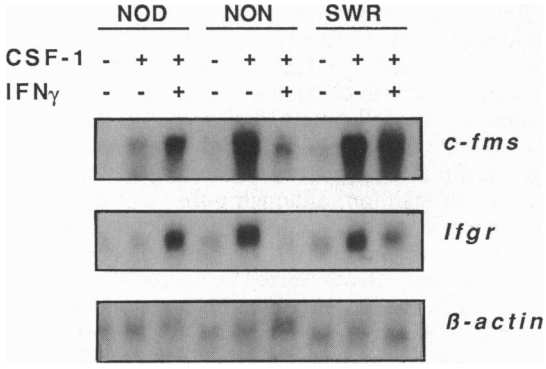

FIG. 2. Northern blot analysis of c-*fms*, *Ifgr*, and β-actin transcript levels in Mφs derived from NOD, NON, and SWR bone marrow cells cultured for 4 days in CSF-1 in the presence and absence of IFN-γ. +, Presence of compound; −, absence of compound.

marrow cultured in CSF-1 alone. However, the addition of IFN-γ greatly decreased c-*fms* transcript levels in CSF-1-stimulated NON marrow and did not affect c-*fms* transcript levels in CSF-1-stimulated SWR marrow (Fig. 2). β-actin transcript levels shown in Fig. 2 indicate that equivalent amounts of RNA were present in each sample.

CSF-1 stimulation normally primes Mφs to be activated by IFN-γ by up-regulating expression of IFN-γ receptor (*Ifgr*) locus on mouse chromosome 10 (25, 26). We therefore analyzed whether the aberrant IFN-γ responses of CSF-1 elicited Mφs from NOD mice was associated with decreased *Ifgr* expression. As expected, high levels of *Ifgr* transcripts were detected in marrow cells of diabetes-resistant NON and SWR mice cultured in CSF-1 alone. *Ifgr* transcript levels were much lower in NON and SWR marrow cells cultured in a combination of CSF-1 and IFN-γ than in marrow cells from these strains cultured in CSF-1 alone (Fig. 2). In contrast, *Ifgr* expression was not up-regulated in CSF-1-elicited Mφs from NOD mice. However, *Ifgr* transcript levels in NOD marrow cells cultured in a combination of CSF-1 and IFN-γ were aberrantly increased to levels equivalent to that of NON and SWR marrow cultured in CSF-1 alone (Fig. 2). Collectively, these results indicate that the reduced ability of NOD mice to generate functionally mature Mφs from hematopoietic precursors is associated with anomalous regulation of RNA transcripts encoding the CSF-1 and IFN-γ receptors.

Analysis of PKC Activity in Bone Marrow-Derived Mφs from NOD Mice and Diabetes-Resistant Strains. CSF-1 stimulates Mφ development by inducing PKC activity via a pertussis toxin-sensitive guanine nucleotide binding protein coupled to the c-fms receptor (27–29). Similarly, IFN-γ functionally activates Mφs in a PKC-dependent manner (30, 31). As described above, defects in the development and activation of Mφs from NOD bone marrow are associated with dysregulated c-*fms* and *Ifgr* expression. Therefore, we compared the time course and magnitude of PKC induction in Mφs derived from NOD marrow cultured with CSF-1 in the presence and absence of IFN-γ to that of similarly derived Mφs from diabetes-resistant NON and SWR control mice.

As shown in Fig. 3, the levels of PKC activity were indistinguishable in NOD, NON, and SWR bone marrow cells cultured for 1 day in CSF-1 alone. However, by culture day 2, PKC activity had increased by ≈4-fold in CSF-1-stimulated bone marrow cells from diabetes-resistant NON and SWR control mice. In contrast, PKC activity only increased slightly in NOD bone marrow cells cultured for 2 days in CSF-1 (Fig. 3). By culture day 4, CSF-1-stimulated PKC activity in NON and SWR bone marrow cells had returned to levels equivalent to that of NOD. Similar results were observed when bone marrow cells from these three strains were cultured in a combination of CSF-1 and IFN-γ. The only difference noted was that even on culture day 1, PKC activity was significantly higher in Mφs derived from NON and SWR bone marrow than in similarly derived Mφs from NOD bone marrow (Fig. 3). Thus, even though the combination of CSF-1 and IFN-γ up-regulated c-*fms* and *Ifgr* transcript levels in NOD Mφs to levels induced in control Mφs by CSF-1 alone (Fig. 2), the ability of these receptors to induce PKC activity remained low in NOD Mφs. This reduced ability to activate PKC-coupled second messenger pathways, in turn, could account for the inability of this combination of cytokines to induce the differentiation of functionally mature Mφs from NOD bone marrow.

Diabetogenic Significance of Impaired APC Development. As assessed by more normal IL-1 secretory responsiveness (Fig. 1), Mφs derived from NON.*H-2*^{g7} marrow cultured in a combination of CSF-1 and IFN-γ were functionally more mature than similarly derived Mφs from NOD mice. IL-1 supplementation *in vitro* circumvents the inability of APCs from NOD mice to activate immunoregulatory T cells in a

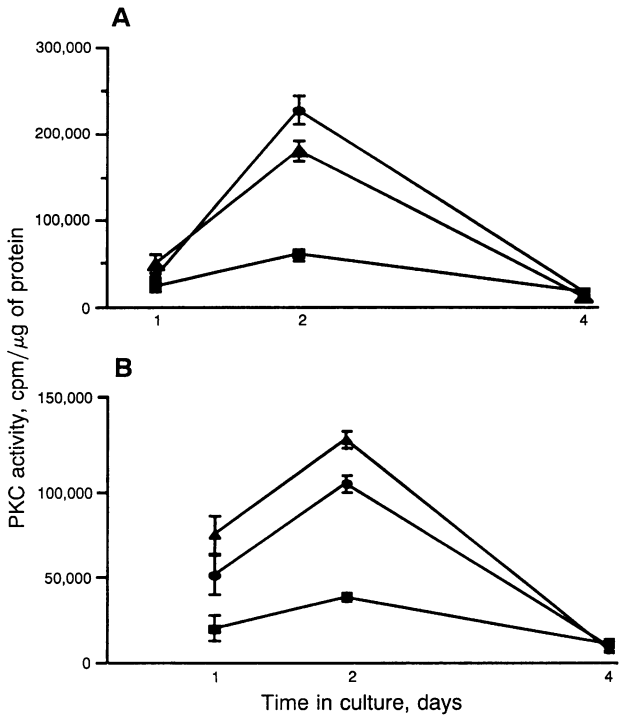

FIG. 3. Comparison of the kinetics and magnitude of PKC induction in Mφs derived from NOD marrow cultured with CSF-1 (*A*) or CSF-1 plus IFN-γ (*B*) to that of similarly derived Mφs from diabetes-resistant NON and SWR control mice. Data represent the cpm (mean ± SEM) of triplicate cultures corrected for nonspecific ^{32}P incorporation obtained in the absence of the PKC-specific synthetic substrate and for the protein concentration of the reaction supernatant. ■, NOD/Lt; ●, NON/Lt; ▲, SWR/Bm.

syngeneic mixed-lymphocyte reaction, and IL-1 administration *in vivo* prevents diabetes (5, 8). Accordingly, we hypothesized that despite expressing the diabetogenic *H-2*^{g7} MHC haplotype, the more functionally mature Mφs generated from NON.*H-2*^{g7} congenic marrow might be capable of blocking the development of diabetogenic T cells from NOD marrow. (NOD × NON)F_1 females were lethally irradiated and reconstituted at 4 weeks of age with either a 1:1 mixture of NOD and NON.*H-2*^{g7} bone marrow cells or with NOD bone marrow cells alone. As expected from previous studies (3, 4), the diabetes-resistance characteristic of unmanipulated F_1 females was abrogated by reconstitution with NOD bone marrow alone, since four of six recipients developed diabetes by 40 weeks of age (Table 1). However, none (zero of six) of the irradiated F_1 hybrids reconstituted with a 1:1 mixture of NOD and NON.*H-2*^{g7} bone marrow cells became diabetic over the same period. Chimerism within the Mφ population could not be assessed due to the absence of polymorphic cell surface markers distinguishing NOD from NON.*H-2*^{g7} marrow-derived Mφs. However, T-cell chimerism could be assessed using the polymorphic Thy-1 alloantigen to distinguish Thy-1.2$^+$ T cells derived from NOD marrow from the Thy-1.1$^+$ T cells derived from NON.*H-2*^{g7} marrow (32). Therefore, splenocytes from each class of chimera were typed by two-color flow cytometric analysis with Thy-1.2- and Thy-1.1-specific monoclonal antibodies to estimate the percentage of leukocytes, including APCs, that had developed from each marrow population. By using these criteria, virtually all of the leukocytes (96.3 ± 1.1%) were found to be of NOD marrow origin in F_1 hybrids reconstituted with NOD marrow alone. In contrast, 63.9 ± 3.0% of the leukocytes were of NOD origin, and 36.2 ± 3.0% of the leukocytes were of NON.*H-2*^{g7} origin, in F_1 hybrids reconstituted with a 1:1 mixture of NOD and NON.*H-2*^{g7} marrow. These results suggest that NON.*H-2*^{g7} marrow-derived APCs expressing non-MHC-linked diabetes-resistance genes are providing a function absent in standard NOD APCs that is necessary for preventing the development or activation of β-cell autoreactive T cells.

Table 1. Functionally mature APCs expressing the diabetogenic *H-2*^{g7} MHC haplotype can block the development of β-cell-autoreactive T cells from NOD bone marrow

Marrow donor(s)	No. mice diabetic by 40 weeks/total no. mice	Leukocytes	
		% NOD derived	% NON.*H-2*^{g7} derived
NOD	4/6	96.3 ± 1.1	3.8 ± 1.0
NOD + NON.*H2*^{g7}	0/6	63.9 ± 3.0	36.2 ± 3.0

Lethally irradiated female (NOD × NON)F_1 hybrids were reconstituted at 4 weeks of age with T-cell-depleted NOD bone marrow cells or with a 1:1 mixture of T-cell-depleted NOD and NON.*H-2*^{g7} bone marrow cells. Upon diabetes onset or reaching 40 weeks of age, splenocytes from the chimeras were typed by two-color flow cytometric analysis for the Thy-1 allotype to estimate the percentage of leukocytes derived from each marrow population (mean ± SEM).

DISCUSSION

We have demonstrated (6) that NOD marrow must be stimulated with a combination of CSF-1 and IFN-γ to generate as many phenotypically mature (Mac-3$^+$) Mφs as are generated from the marrow of diabetes-resistant strains stimulated with CSF-1 alone. Recently, Langmuir *et al.* (7) have demonstrated that myeloid cells failed to develop normally from NOD bone marrow stimulated with granulocyte–Mφ colony-stimulating factor, interleukin 3, or interleukin 5. Mφs acquire the ability to produce TNF-α during the early stages of development but do not acquire the ability to produce IL-1β until the latter stages of differentiation (9). In the present study, we found that marrow-derived Mφs from NOD mice and diabetes-resistant SWR mice secreted equivalent levels of TNF-α after LPS stimulation. In contrast to the results of Jacob *et al.* (8), we have also failed to find any differences in TNF-α secretion between LPS-stimulated peritoneal Mφs from NOD mice and diabetes-resistant control strains (data not shown). However, we are in agreement that LPS-stimulated peritoneal Mφs from NOD mice are poor IL-1 producers (5), suggesting that a large proportion of APCs in NOD mice may be incompletely differentiated. This possibility is further supported by our current finding that while Mφs derived from NOD marrow cultured in a combination of CSF-1 and IFN-γ are phenotypically equivalent to similarly derived Mφs from diabetes-resistant strains, these cells remain functionally immature as assessed by a decreased ability to secrete IL-1 after LPS stimulation.

The decreased ability of CSF-1 to induce Mφ development from precursor cells in NOD marrow and to prime these cells to become functionally mature in response to IFN-γ was associated with an inability of this myeloid growth factor to up-regulate c-*fms* (CSF-1 receptor) and *Ifgr* (IFN-γ receptor) expression. In addition, although c-*fms* and *Ifgr* transcript levels were up-regulated in Mφs derived from NOD marrow cultured in a combination of CSF-1 and IFN-γ, these cytokine receptors were characterized by a greatly reduced ability to activate PKC-coupled second messenger pathways. However, impaired APC development and function in NOD mice may result from a generalized PKC activation defect that has pleiotropic effects. Antigen-stimulated T-cell proliferation is also dependent upon activation of PKC-coupled second messenger pathways (33). The decreased ability of NOD thymocytes to proliferate in response to T-cell receptor cross-linking agents (34) has also been associated with an

inability to induce PKC activity. This indicates that NOD mice are characterized by PKC induction defects in more than one cell lineage derived from hematopoietic stem cells. In turn, a generalized PKC induction defect could account for the wide range of aberrant responses to IFN-γ that characterize NOD mice. In addition to failing to become functionally activated as assessed by a decreased ability to secrete IL-1, Mø from NOD mice also aberrantly down-regulate total MHC class I expression after exposure to IFN-γ (6). Exposure to IFN-γ also induces expression of an "occult" MHC class I-like molecule in both Mø and pancreatic β cells from NOD mice (35). The initiation of β-cell destruction requires both $CD4^+$ and $CD8^+$ T cells (22). It is possible that these unusual IFN-γ responses associated with abnormal PKC activation may impair the ability of APCs from NOD mice to stimulate autoreactive MHC class I-restricted $CD8^+$ T cells to an activation state high enough to induce their destruction. These autoreactive T cells may then be efficiently targeted to pancreatic β cells expressing high levels of MHC class I restriction elements.

T-cell mediated autoimmune diabetes in NOD mice results from complex interactions between multiple MHC and non-MHC-linked susceptibility modifiers (2). The findings that APCs expressing a diabetes-resistant MHC haplotype can block the development of diabetogenic T cells from NOD bone marrow (4) and that immunostimulatory treatments up-regulating APC function also block disease (5, 8, 36) indicate that many of these complex polygenic interactions occur at the level of hematopoietically derived APCs. In general, a higher threshold of T-cell activation is required to induce tolerance than is required to trigger T-cell effector responses (10, 12). Thus, genetic defects compromising the ability of APCs to process and/or present antigens efficiently or, alternatively, to provide costimulatory signals may impair the ability of these cells to induce tolerance without fully abrogating their ability to activate effector T-cell function. Indeed, the finding that diabetes is blocked either by intrathymic engraftment of syngeneic β cells into young prediabetic NOD mice (37) or by introduction of purified APCs from pancreatic lymph nodes (38) supports the concept that high concentrations of β-cell autoantigens within the thymus may compensate for the inability of APCs from NOD mice to present these antigens in a manner quantitatively sufficient to induce tolerance. This possibility is strongly supported by our demonstration in a competitive bone marrow chimera system that APCs that express the diabetogenic $H\text{-}2^{g7}$ MHC haplotype but are functionally more mature than NOD APCs can block the development or peripheral activation of diabetogenic T cells. These results provide further evidence that the aberrant immunophenotypes exhibited by NOD APCs are the logical starting point for dissecting the complex interactions between MHC- and non-MHC-linked genes that control diabetogenesis.

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