Aberrant Macrophage Cytokine Production Is a Conserved Feature Among Autoimmune-Prone Mouse Strains

Elevated Interleukin (IL)-12 and an Imbalance in Tumor Necrosis Factor-α and IL-10 Define a Unique Cytokine Profile in Macrophages From Young Nonobese Diabetic Mice

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Cytokines derived from macrophages (Mϕ) play a critical role in the development of type 1 diabetes in the nonobese diabetic (NOD) mouse. Based on earlier findings from lupus-prone strains of inherent cytokine defects in Mϕ, NOD Mϕ were evaluated for intrinsically dysregulated cytokine production with the potential to initiate or exacerbate disease. Endotoxin-activated peritoneal Mϕ from young prediseased NOD mice produced interleukin (IL)-1 and tumor necrosis factor (TNF)-α levels similar to those of Mϕ from a panel of control strains but reduced compared with the congenic diabetes-resistant NOR strain. IL-6 and IL-10 production were similar in NOD and NOR Mϕ, indicating that reduction in NOD IL-1 and TNF-α expression was selective. Nevertheless, the ratio of TNF-α and IL-10 production, a stringent index of normal Mϕ function, distinguished NOD from all normal strains. The most striking feature of NOD Mϕ, however, was their substantially elevated IL-12 production. This response was induced not only by endotoxin but also by bacillus Calmette-Guérin (BCG) and CD40 ligand and was associated with (and likely caused by) the enhanced and prolonged expression of p40 mRNA. Moreover, NOD Mϕ IL-12 expression appeared to be near maximally induced by lipopolysaccharide (LPS) alone, because it was only slightly enhanced by the addition of γ-interferon, a stimulus that substantially elevated LPS-induced IL-12 production in Mϕ from normal strains. Accompanied by a unique profile of TNF-α and IL-10, the dramatic elevation of IL-12 expression by NOD Mϕ reflects intrinsic defects of the innate immune system with the potential to initiate and propagate the pathogenic autoreactive T-helper type 1 response characteristic of type 1 diabetes. ***Diabetes*** **49:1106–1115, 2000**

Several genetic and environmental factors appear to cooperate to precipitate type 1 diabetes, a spontaneous autoimmune disease in humans and in the nonobese diabetic (NOD) mouse (1). NOD mice, like type 1 diabetic patients, develop insulitis, an early infiltration of leukocytes into the pancreas that leads to inflammatory lesions within the islets (1,2). However, overt type 1 diabetes requires the subsequent destruction of the vast majority of insulin-producing islet β-cells, a process mediated by activation of autoreactive T-helper (Th) type 1 CD4$^+$ and, subsequently, CD8$^+$ T-cells (1,3–5). Although the genetic defects responsible for the different stages of type 1 diabetes have not yet been identified, there has been extensive characterization of several chromosomal regions (*Idd* loci) involved in type 1 diabetes that clearly supports the multigenic nature of this disease (2). Thus, the unique NOD major histocompatibility complex (MHC) H-2^{g7} haplotype contained in *Idd1* is necessary, but not sufficient, for diabetes to develop (6,7), and several non–MHC-linked *Idd* loci have been demonstrated to contribute substantially to the development of type 1 diabetes (2).

Some clues to identifying the type 1 diabetes–promoting genes within these non–MHC-linked *Idd* loci have come from studies that demonstrated aberrant antigen-presenting cell (APC) and accessory cell activities by macrophages (Mϕ) from NOD mice. In addition to the association of the *Idd1* locus with aberrant APC function, which may lead to the failure to cause deletion of β-cell–specific autoreactive T-cells (1,2,8), non–MHC-linked *Idd* loci contribute to defective immunomodulatory functions in Mϕ and other APCs, including defective stimulation of immunoregulatory T-cells

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Received for publication 25 June 1999 and accepted in revised form 10 March 2000.

APC, antigen-presenting cell; BCG, bacillus Calmette-Guérin; CD40L, CD40 ligand; ELISA, enzyme-linked immunosorbent assay; HBSS, Hanks' balanced salt solution; IFN-γ, γ-interferon; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibodies; MHC, major histocompatibility complex; Mϕ, macrophages; rMu, recombinant murine; Th, T-helper; TNF, tumor necrosis factor.

(9–12). Aberrant Mϕ function in models not only of diabetes but also of lupus has been associated with reduced production of interleukin (IL)-1 and tumor necrosis factor (TNF)-α (9,11–15). In fact, the prominent role of cytokines derived from Mϕ in type 1 diabetes has been demonstrated by the modulation of disease after administration of TNF-α or IL-1 to NOD mice (14). Of importance both therapeutically and regarding the mechanism underlying disease progression, treatment with TNF-α early in life leads to exacerbation of disease, whereas later administration of either TNF-α or IL-1 prevents disease (14). It is noteworthy that a similar dichotomy develops in response to IL-10 treatment (16), suggesting the premise that the contribution of a specific cytokine at one stage of disease may be quite different from its role during a subsequent pathological event. Importantly, analysis of the TNF-α gene in NOD mice indicates that the aberrant pattern of expression of this cytokine is likely due to defective regulation of gene expression rather than alterations in the structural gene itself (17).

Another cytokine derived from Mϕ, IL-12, is required for the development of Th1 responses (18) and appears to contribute to disease in NOD mice by enhancing γ-interferon (IFN-γ) production and subsequent β-cell destruction by infiltrating Th1 $CD4^+$ and $CD8^+$ T-cells (19). IL-12 mRNA expression in pancreatic islets in NOD mice increases with β-cell destruction (4), whereas in vivo neutralization of IL-12 blocks disease (20,21). From a broader perspective, recent studies have revealed that cytokines produced by cells of the innate immune system, especially Mϕ and natural killer cells, play a critical role in both initiating and shaping the immune response (22). These observations suggest that some *Idd* susceptibility loci may be associated with the regulation of cytokine expression, especially that by Mϕ.

Previous studies exploring cytokine production by Mϕ from NOD mice before the onset of disease have not always reported consistent results regarding TNF-α and IL-1 expression (9–12,14). Moreover, expression of other cytokines, such as IL-6, IL-10, and IL-12, has not been studied in NOD Mϕ. To more fully characterize intrinsic abnormalities in Mϕ cytokine production (i.e., aberrant patterns expressed before the onset of any disease signs) and, in particular, to examine those cytokines that are likely to contribute to diabetogenesis, we evaluated levels of TNF-α, IL-1, IL-6, IL-10, and IL-12 produced by endotoxin-activated Mϕ. We compared the activity of Mϕ from young prediseased NOD mice with those of a panel of control strains, including the MHC-compatible NOR strain, which is largely congenic with the NOD strain (12). Our results demonstrate that the levels of TNF-α and IL-1 produced by NOD Mϕ are reduced relative to NOR Mϕ. Levels of TNF-α, IL-1, and IL-10 produced by NOD Mϕ are each within, but at the lower end of, the range produced by Mϕ from a panel of normal strains. More stringent analysis revealed that the ratio of TNF-α to IL-10 produced by NOD Mϕ is distinct from that produced by Mϕ from all normal strains tested, including NOR. However, the most striking characteristic of NOD Mϕ is that their IL-12 production far exceeds that of Mϕ from any other strain and may thus predispose and contribute to the Th1-mediated pathology of diabetes. The characterization of the cytokine network intrinsic to Mϕ from normal and autoimmune-prone mice may aid in identifying genes that contribute to the development of autoimmunity and provide insights into regulation of the cellular events associated with these diseases.

RESEARCH DESIGN AND METHODS

Animals. For the study, 3- to 4-week-old BALB/c, A/J, C57BL/6, C57BL/10, C3H/OuJ, NOR, and NOD male mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained for 1 week after arrival in a pressurized (1-way flow) room.

Reagents. Recombinant murine (rMu) TNF-α (10^7 U/mg) and rMu IL-6 (1 µg/ml) were purchased from PharMingen (San Diego, CA). rMu IFN-γ (10^4 U/mg) was provided by Genentech (San Francisco, CA), rMu IL-12 (198 µg/ml) was provided by Dr. Victor H. Van Cleave, Bioanalytical Sciences Department, Genetics Institute (Cambridge, MA); rMu IL-10 (1.4×10^4 U/ml) was provided by Dr. Kevin Moore, DNAX (Palo Alto, CA); and rMu CD40 ligand (CD40L) was provided by Dr. Marilyn R. Kehry, Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). RPMI-1640 medium with glutamine (catalog number 12-702F) was supplemented with 0.5% HEPES (catalog number 17-737), 1% penicillin/streptomycin solution (catalog number 17-602E), and 5% fetal bovine serum (all components of medium from BioWhittaker, Walkersville, MD) and was used for culturing Mϕ. Hanks' balanced salt solution (HBSS) (BioWhittaker) was used for cell washing. No endotoxin (<10 pg/ml) was detected using the Limulus amoebocyte assay in any of the reagents and media described above. Lipopolysaccharide (LPS) (*Escherichia coli*: 0111:B4; Sigma, St. Louis, MO) and heat-killed bacilli Calmette-Guérin (BCG) were stored at –70 and 4°C, respectively, at 2 mg/ml in H_2O and diluted immediately before use.

Macrophage isolation and culturing. Peritoneal exudate Mϕ were obtained by peritoneal lavage with cold HBSS 4 days after an injection (2 ml i.p.) of 4% thioglycollate broth (Remel, Windsor, CT; catalog number 07178). Cells were pooled from at least 3 mice per strain, washed, resuspended in fresh medium, and seeded at 2×10^5 cells in 100 µl per well of 96-well flat-bottom tissue culture-treated plates (Costar, Cambridge, MA; catalog number 3596). Cells were incubated for 2 h at 37°C, 5% CO_2, in a humidified chamber to allow Mϕ to adhere and spread. Nonadherent cells were removed by adding 200 µl of warmed HBSS to each well, resuspending nonadherent cells by moderately tapping the plate, and flicking the plate to discard the nonadherent cells. This washing was performed 3 times, after which 50 µl of medium was immediately added to each well. Remaining cells were >98% Mϕ, as assessed by morphologic examination and nonspecific esterase staining. To ensure that equal numbers of adherent Mϕ among strains remained after washing, nonadherent cells from washes of single wells from each strain were routinely counted and showed no differences among strains. Cytokines, LPS, or medium was added to each Mϕ culture to yield a final volume of 200 µl. Culture-conditioned medium was collected at appropriate times in fresh plates and stored at –20°C for assessment of all cytokines except IL-1. Conditioned medium was collected in a sequential fashion (e.g., 0–16, 16–24, and 24–36 h) with change of medium and fresh LPS at each time point, which permits quantitation of cytokine levels independent of levels produced during an earlier period. IL-1 levels were assessed in Mϕ lysates because substantial IL-1 is retained in the cell (23). Mϕ lysates were prepared by adding 200 µl medium to each well of Mϕ monolayers after conditioned medium was collected. Cells were subjected to 3 rapid cycles of freeze-thawing at –70 and 37°C, respectively, and stored at –20°C. The 3 culturing periods used for the kinetic assessment of each cytokine correlate to early, intermediate, and late periods that collectively encompass virtually all cytokine production. For example, >95% of the total amount of TNF-α is produced within 24 h, whereas this fraction of IL-6 and IL-12 requires 36 h of stimulation, and that of IL-1 requires 72 h of stimulation (data not shown).

Assessment of cytokines. IL-1 bioactivity in Mϕ lysates was measured in the D10.G4 bioassay as previously described (23,24), and TNF-α levels in medium conditioned with Mϕ were assessed using the WEHI-164 bioassay as previously described (24,25). Alamar Blue solution (AccuMed International, Westlake, OH; catalog number 00-100) was used to assess cell viability in both bioassays in which 1 U of IL-1 or TNF-α activity was defined as the titer of cell lysates or culture-conditioned medium, respectively, that maintained 50% of maximal cell viability. IL-6 and IL-10 levels in medium conditioned with Mϕ were assessed by enzyme-linked immunosorbent assay (ELISA) (monoclonal antibodies [mAb] from PharMingen; anti–IL-6 mAb, catalog number 18071D and 18082D; anti–IL-10 mAb, catalog number 18141D and 18152D). IL-12 levels in medium conditioned with Mϕ were assessed by a Mu IL-12–specific ELISA (a gift from Dr. Victor H. Van Cleave). Briefly, 2 rabbit polyclonal anti–rMu IL-12 antibody preparations, R03B03 (3 µg/ml) and B03B02 (biotinylated, 1:500 dilution), that recognize the IL-12 heterodimer (p70) were used for capture and detection, respectively, along with avidin-labeled peroxidase (Sigma, catalog number A-3151) and ABTS solution (Sigma, catalog number A-1888) for development. Conditioned medium derived from Mϕ was undiluted when added to the IL-10

and IL-12 ELISAs and diluted at least 2-fold upon addition to the IL-6 ELISA, and rMu cytokines were used to generate the respective standard curves.

Analysis of IL-12 p40 and p35 subunit mRNA expression by RNase protection assay. Thioglycollate-elicited peritoneal exudate cells (10^7) were plated from each mouse strain on 75-cm^2 plastic tissue culture–treated dishes (Nalge Nunc International, Roskilde, Denmark), and Mϕ were allowed to adhere for 2 h at 37°C, 5% CO_2. Nonadherent cells were washed away and adherent Mϕ were activated with LPS (100 ng/ml) for 4, 8, 12, and 24 h, at which time total RNA was extracted using TRIzol Reagent (Gibco, Grand Island, NY), as specified in the manufacturer's instructions. RNA (10 μg) from each sample was reprecipitated and used in the RNase protection assay, which included probes for the p40 and p35 subunits of IL-12 (PharMingen). Samples were separated on a 7-mm sequencing gel and visualized by autoradiography. RNA was quantified and normalized to GAPDH control RNA using the ImageQuant densitometry program.

Statistical analysis. All means and SE in figures were calculated from triplicate values; the Student's *t* test was used to compare mean values. In Fig. 3, the relationship between TNF-α and IL-10 produced by Mϕ from normal strains was determined through linear regression. As a measure of divergence of NOD Mϕ from this regression, the predicted TNF-α level for NOD Mϕ was then compared with the observed value using a 2-tailed *t* test.

RESULTS

TNF-α and IL-1 production by NOD Mϕ is reduced compared with NOR Mϕ. To characterize the cytokine expression profile by NOD Mϕ, we first assessed cytokines that have been shown to play a role in diabetogenesis in the NOD mouse, namely, TNF-α and IL-1. We compared levels of intracellular IL-1 and secreted TNF-α bioactivity produced by Mϕ from NOD mice and from the 5 age-matched control strains BALB/c, A/J, C57BL/6, C57BL/10, and C3H/OuJ, as well as the congenic NOR strain. Mϕ were activated with LPS for 16 h and assessed for intracellular IL-1. A period of 12 h (the period of maximum TNF-α production [24]) was used to prepare conditioned medium to analyze for TNF-α. Our findings revealed that NOD Mϕ produced 4- to 5-fold less IL-1 (Fig. 1) and 7- to 8-fold less TNF-α (Table 1) than NOR Mϕ. IL-1 was also studied at later times, i.e., at 24–72 h, and a similar pattern was noted (data not presented), indicating that the differences observed are not due merely to a shift in the kinetics of cytokine production. However, NOD IL-1 and TNF-α levels were within the range of the other control strains, whereas NOR Mϕ are, like A/J Mϕ, high producers of both cytokines. The reduced IL-1 and TNF-α expression by Mϕ from NOD mice is not associated with a reduction in viability or metabolic activity compared with the other strains, as measured by conversion of the redox reagent Alamar Blue (data not shown). Considering the degree of genetic identity between the NOD and NOR congenic strains, these data reveal that reduced IL-1 and TNF-α levels are associated with the limited genetic differences that, together, lead to disease in the NOD mouse.

IL-6 and IL-10 are produced at similar levels by NOD and NOR Mϕ. IL-6 was next assessed to determine whether proinflammatory cytokines were, as a group, distinctly regulated in NOD Mϕ compared with NOR Mϕ. Mϕ were activated for 12 h, and levels of secreted IL-6 were determined by ELISA. In contrast to IL-1 and TNF-α, IL-6 levels were more consistent among control strains, varying only ~4-fold among Mϕ from all strains tested (Table 1), which is consistent with our earlier observations on the expression of these cytokines (24). Interestingly, in contrast to TNF-α and IL-1, IL-6 expression was similar in NOR and NOD Mϕ (Table 1), as was the case at later times (12–24 and 24–36 h; data not shown). These data demonstrate that Mϕ from NOD mice do not display a global dysregulation of proinflammatory cytokine production relative to diabetes-resistant NOR mice.

Production of IL-10 was studied both to provide a more comprehensive evaluation of Mϕ function as well as to test the possibility that the reduced expression of IL-1 and TNF-α by NOD versus NOR Mϕ might reflect a concomitant overexpression of this anti-inflammatory cytokine. Mϕ were activated with LPS for 12 h (the period of maximum IL-10 pro-

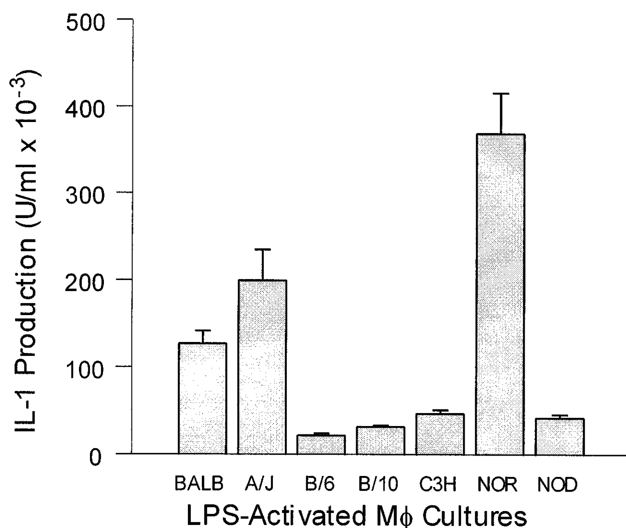

FIG. 1. IL-1 production by peritoneal Mϕ from NOD mice. Two × 10^5 thioglycollate-elicited peritoneal Mϕ from 6 diabetes-resistant strains (BALB/c, A/J, C57BL/6, C57BL/10, C3H/OuJ, and NOR) and the diabetes-prone NOD strain were activated with 100 ng/ml LPS, and cell monolayers were harvested at 16 h for cell lysate preparation and assessed for IL-1 bioactivity using the D10.G4 bioassay. Values are the mean and SE of triplicate cultures from 1 experiment representative of at least 4 experiments per strain.

TABLE 1
Relative cytokine production by Mϕ from normal and NOD strains

	Relative cytokine production		
	TNF-α	IL-6	IL-10
n	7	3	6
Mϕ strain			
A/J	7.18 ± 1.24*	1.67 ± 0.07	0.09 ± 0.03*
C57Bl/6	0.46 ± 0.09*	1.32 ± 0.16	1.42 ± 0.22*
C57BL/10	0.81 ± 0.20	2.05 ± 0.68*	0.95 ± 0.26
C3H/OUJ	0.43 ± 0.11*	1.22 ± 0.31	1.60 ± 0.16*
NOR	6.95 ± 2.12*	5.10 ± 0.84*	0.14 ± 0.05*
NOD	0.95 ± 0.41	4.96 ± 1.67*	0.30 ± 0.08*

Means ± SE of normalized values from several experiments (i.e., *n*) are presented. Two × 10^5 peritoneal Mϕ from each murine strain were cultured with LPS (100 ng/ml) for 12 h, and conditioned medium was analyzed for TNF-α levels by WEHI-164 cell bioassay and for IL-6 and IL-10 levels by ELISA. Values of cytokine production for each strain were divided by values from BALB/c in each experiment to obtain normalized values of cytokine production. BALB/c cytokine production from representative experiments for TNF-α was 944 ± 59 U/ml, for IL-6 was 44.5 ± 2.2 ng/ml, and for IL-10 was 0.46 ± 0.05 U/ml. *Significantly ($P < 0.01$) different from BALB/c values.

duction [24]), and conditioned medium was analyzed for IL-10 by ELISA (Table 1). IL-10 expression displayed as much variability among strains as did TNF-α (i.e., ~20-fold), and NOD IL-10 levels were within the range produced by control strains. Like IL-6, levels of IL-10 were similar in NOR and NOD Mϕ, and this pattern did not change over time in culture. These results indicate that production of neither IL-10 nor IL-6 is sensitive to the same regulatory factors that control IL-1 and TNF-α, and, moreover, that IL-10 is unlikely to contribute to the reduced expression of TNF-α and IL-1 in NOD Mϕ. With the exception of the pattern of IL-6 expression, these findings were quite similar to our previous observations with Mϕ from the lupus-prone MRL/++ and NZB/W F1 strains (24), suggesting a remarkable conservation of aberrant Mϕ cytokine production in multigenic models of both systemic and organ-specific autoimmunity.

Reduced TNF-α production by NOD Mϕ is substantially independent of endogenous IL-10. The previous data addressed the independent expression of individual cytokines. In the next two sections, we explore the interrelation of 2 of them—TNF-α and IL-10. To verify that the autocrine inhibitory action of IL-10 was not responsible for reduced TNF-α production by NOD Mϕ, TNF-α production was assessed in the presence or absence of anti–IL-10 mAb (Fig. 2). The neutralization of IL-10 modulated TNF-α production by Mϕ from all strains tested, consistent with their endogenous IL-10 levels: greater enhancement of TNF-α production occurred in Mϕ that produced high (e.g., BALB/c, B/6, B/10, and C3H/OuJ) or intermediate (NOD) IL-10 levels than in Mϕ that produced low amounts of IL-10 (A/J and NOR). Neutralization of IL-10 did not restore NOD Mϕ TNF-α levels to those of NOR Mϕ (Fig. 2), although it reduced the extent of the defect from ~40- to 10-fold. These findings do not indicate a major role for IL-10 in establishment of the TNF-α defect in NOD Mϕ but are compatible with the modest (2-fold) elevated IL-10 levels produced by NOD Mϕ compared with NOR Mϕ, as noted in Table 1.

The precise balance of IL-10 and TNF-α levels demonstrated by Mϕ from control strains is not conserved in NOD Mϕ. To this point, TNF-α and IL-1 expression by Mϕ from NOD mice have been characterized as aberrant only within the context of the genetic background common to NOR and NOD. However, the pattern of cytokine production by NOD Mϕ can also be evaluated in the broader context of a panel of control strains by determining the balance between TNF-α and IL-10. We have previously shown that this relationship is stringently regulated among Mϕ from the 3 normal strains BALB/c, A/J, and C57BL/6 (24). As shown in Table 1, whereas there are substantial differences in absolute levels of TNF-α and IL-10 produced by Mϕ from a total of 5 normal strains (including NOR) compared with BALB/c, IL-10 levels are in each case inversely proportional to the level of TNF-α produced by Mϕ from the same strain. In fact, when relative TNF-α levels are plotted against relative IL-10 levels (data, normalized to BALB/c, from Table 1), a rigorous

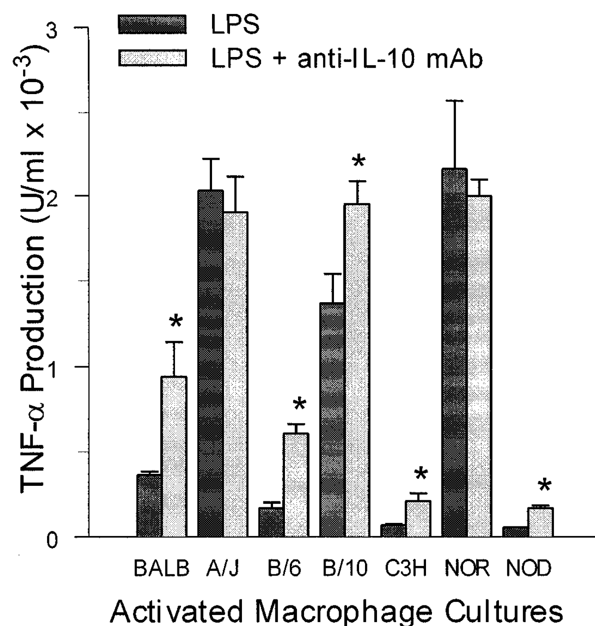

FIG. 2. IL-10 autocrine regulation of TNF-α production by Mϕ from control and NOD strains. Two × 10^5 thioglycollate-elicited peritoneal Mϕ from 6 control strains (BALB/c, A/J, C57BL/6, C57BL/10, C3H/OuJ, and NOR) and the diabetes-prone NOD strain were activated with 100 ng/ml LPS in the presence or absence of anti–IL-10 mAb (1 µg/ml) or rat IgG_1 isotype control mAb (not shown), and conditioned culture medium was collected at 12 h and assessed for TNF-α levels. Each bar represents the mean and SE of triplicate values from 1 experiment representative of at least 3 experiments per strain. Isotype control mAb at 0.1 and 1 µg/ml had no significant effect on TNF-α production by Mϕ from any strain. *Significantly ($P < 0.05$) different from control values of cultures with LPS alone.

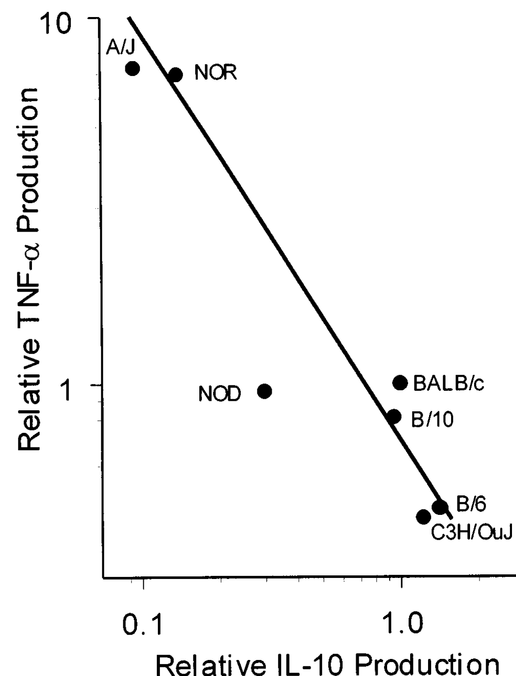

FIG. 3. Relationship of IL-10 and TNF-α levels in cultures of Mϕ from NOD and control strains. Two × 10^5 thioglycollate-elicited peritoneal Mϕ from 6 control strains (BALB/c, A/J, C57BL/6, C57BL/10, C3H/OuJ, and NOR) and the diabetes-prone strain NOD were activated with 100 ng/ml LPS; conditioned culture medium was collected at 12 h and assessed for TNF-α and IL-10 levels. Cytokine levels from each Mϕ strain were normalized to BALB/c Mϕ values. Each point represents the mean of normalized values from 7 experiments per strain for TNF-α production and 6 experiments per strain for IL-10 production (data are from Table 1, which also provides SE values). Linear regression analysis for control strain values demonstrated a correlation coefficient (r^2) of 0.90; inclusion of the NOD value reduced r^2 to 0.64.

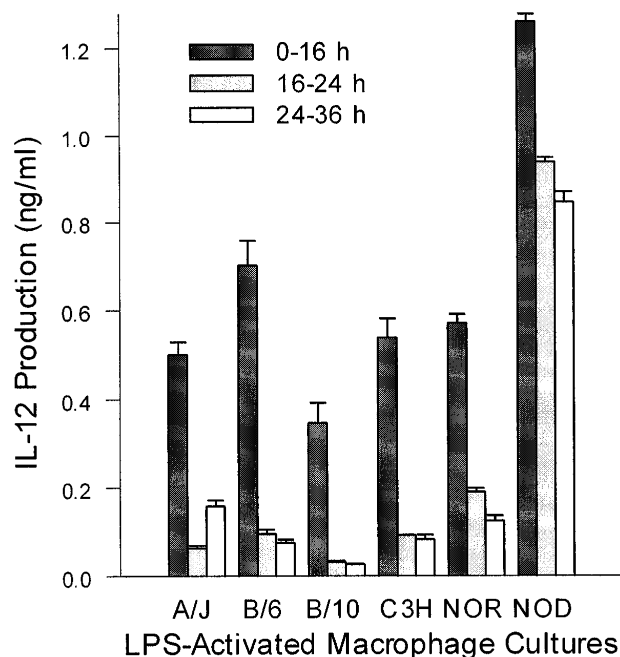

FIG. 4. Elevated expression of IL-12 by LPS-stimulated NOD Mϕ. Two × 10^5 thioglycollate-elicited peritoneal Mϕ from 5 control strains (A/J, C57BL/6, C57BL/10, C3H/OuJ, and NOR) and the diabetes-prone NOD strain were activated with 100 ng/ml LPS and incubated for 3 sequential intervals, as indicated. At the end of each interval, conditioned medium was removed and cultures replenished with fresh medium and LPS. Mean levels of IL-12 and SE of triplicate cultures from 1 experiment representative of at least 4 experiments per strain are shown.

reciprocal relationship between these 2 cytokines, determined by regression analysis, is noted (correlation coefficient 0.90; Fig. 3). Whereas relative NOD TNF-α and IL-10 values are each within the normal range, they are both found at the lower end of this range (Table 1), a finding inconsistent with their normally stringent inverse relationship. Thus, the NOD Mϕ TNF-α/IL-10 ratio was displaced from the regression line (Fig. 3). Because IL-10 levels control TNF-α expression but not vice versa (24), this regression was used to ***predict*** an NOD TNF-α value based on the observed NOD IL-10 level. This predicted TNF-α level was then compared with the actual NOD TNF-α level using a two-tailed *t* test and revealed that the variance of the NOD value from the regression characterizing the 6 normal strains was statistically significant ($P < 0.05$). This variance was also reflected in the correlation coefficient (r^2 value), which was reduced from 0.90 to 0.64 when the NOD values were included in the regression analysis.

Elevated expression of IL-12 by NOD Mϕ is induced by diverse stimuli. It is well established that type 1 diabetes in NOD mice is associated with the development of a pathogenic Th1 response, which can be exacerbated by injections of IL-12 or blocked by in vivo neutralization of this cytokine (20). Thus, we evaluated IL-12 production by Mϕ, which are a major source of this cytokine. Mϕ from control and NOD mice were activated with LPS, and conditioned medium was collected in 3 sequential intervals to quantitatively and qualitatively evaluate IL-12 production. Mϕ from NOD mice produced more IL-12 during the initial culture period than did Mϕ from any other strain, and this difference became even more pronounced with time (Fig. 4). IL-12 was expressed at similar levels by Mϕ from each control strain during the early interval, and IL-12 diminished during the intermediate and late intervals to levels that ranged from 10 to 30% of the initial values. However, IL-12 production by NOD Mϕ during the intermediate and late intervals was maintained at ~70% of initial levels (Fig. 4). Thus, NOD Mϕ IL-12 production, which was initially 2- to 3-fold greater than that of Mϕ from control strains, became 5- to 25-fold greater than that of the other strains during later periods of culture. Moreover, elevated IL-12 expression by NOD Mϕ was not limited to activation by

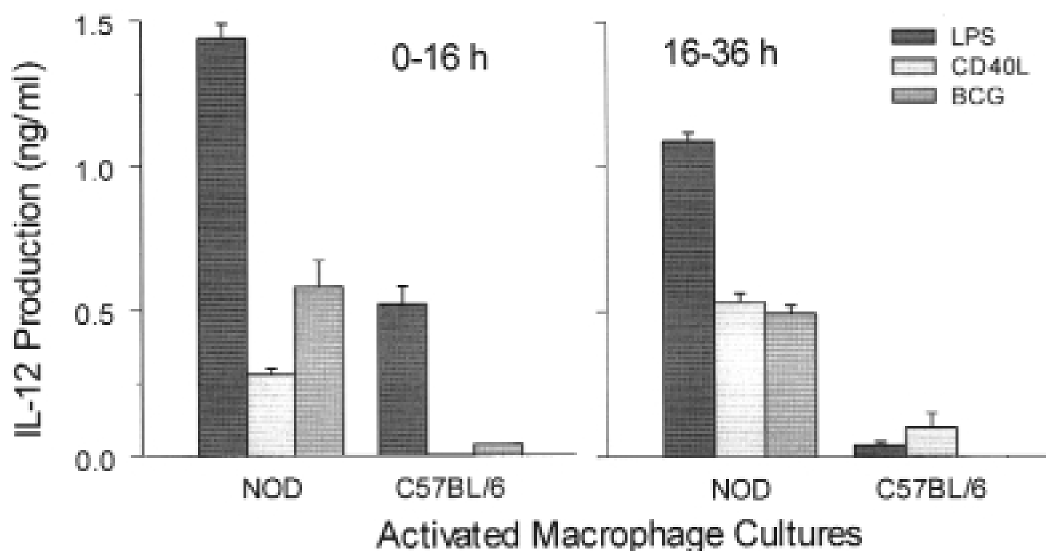

FIG. 5. Elevated expression of IL-12 by BCG- and CD40L-induced NOD Mϕ. Two × 10^5 thioglycollate-elicited peritoneal Mϕ from the C57BL/6 control strain and the diabetes-prone NOD strain were activated with LPS (100 ng/ml), BCG (2 μg/ml), or CD40L (2 μg/ml) and incubated for 2 sequential intervals in which conditioned medium was removed and cultures replenished with fresh medium and stimulus. Mean levels of IL-12 and SE of triplicate cultures from 1 experiment representative of at least 3 experiments per strain are shown.

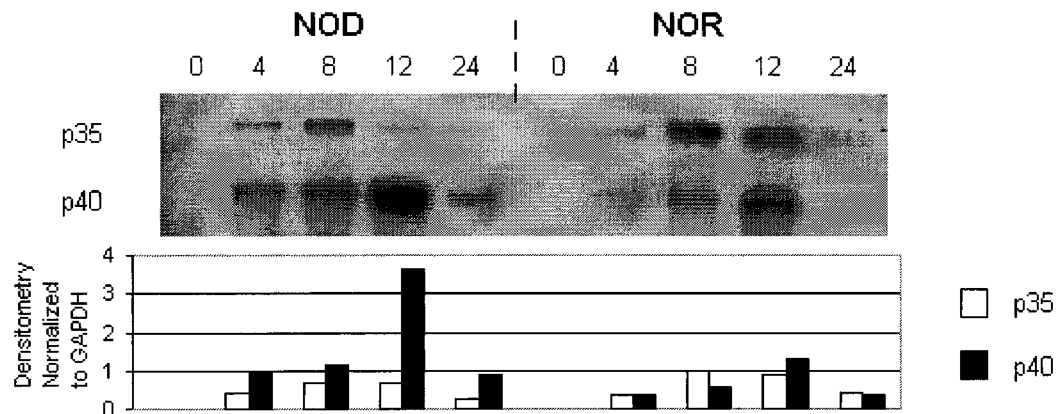

FIG. 6. Elevated expression of the IL-12 p40 subunit gene by NOD Mϕ. Thioglycollate-elicited peritoneal exudate cells (10^7) from NOD and NOR Mϕ were activated with LPS (100 ng/ml) for 4, 8, 12, and 24 h, at which time total RNA was extracted and mRNA levels of the p40 and p35 subunits of IL-12 were assessed in an RNase protection assay. Samples were separated on a sequencing gel and visualized by autoradiography (upper panel). RNA was quantified by densitometry and normalized to GAPDH control (lower panel). Results represent 1 of 3 experiments that had similar results.

LPS, as demonstrated by the response to heat-killed mycobacterium (BCG) and CD40L (Fig. 5).

Elevated expression of IL-12 p40 mRNA in NOD Mϕ. We determined whether elevated IL-12 production by NOD Mϕ might be associated with augmented transcript levels for the p35 or p40 subunits of the functional IL-12 receptor. Using the RNase protection assay, p40 and p35 mRNA levels were assessed in NOR and NOD Mϕ at 4, 8, 12, and 24 h after LPS activation (Fig. 6). Unactivated Mϕ did not express either subunit; LPS induced p35 and p40 mRNA expression by Mϕ from both strains as early as 4 h and induced maximal expression of p35 between 8 and 12 h and of p40 by 12 h. However, whereas NOR and NOD Mϕ expressed similar levels of p35 at each time point (determined by normalizing p35 levels to GAPDH levels within each sample; Fig. 6, lower panel), NOD Mϕ consistently expressed 2 to 3 times more p40 mRNA than NOR Mϕ (Fig. 6). A more striking trend was noted by comparing the balance of p35 and p40 mRNA in Mϕ from each strain. In NOR Mϕ, p40 mRNA levels exceeded those of p35 only at 1 time point (12 h) and then by <40%. In contrast, NOD Mϕ p40 mRNA expression exceeded that of p35 at all time points: by ~2-fold at 4 and 8 h and by 4- to 5-fold at 12 and 24 h. These results suggest that the preferential induction or stabilization of p40 mRNA is likely to account for the elevated IL-12 production by NOD Mϕ, which is consistent with the known role of p40 in regulating IL-12 heterodimer production (18).

Augmentation of Mϕ IL-12 expression by IFN-γ reveals that NOD Mϕ produce near-maximal levels of IL-12 in response to LPS alone. The Th1-derived cytokine, IFN-γ, is known to dramatically potentiate LPS-stimulated Mϕ IL-12 production (28). We reasoned that if IFN-γ–mediated signaling was intact in the NOD Mϕ, a blunted capacity of IFN-γ to further enhance NOD Mϕ IL-12 production would provide evidence that this cell was already producing IL-12 at, or near, maximal levels. Thus, we evaluated LPS-induced IL-12 production (as well as induction of TNF-α as a control for IFN-γ responsiveness) in the presence or absence of IFN-γ. IFN-γ treatment potentiated LPS-stimulated IL-12 levels in Mϕ from all strains tested during both the early (0–16 h) and late (16–24 h) intervals (Fig. 7); the degree of responsiveness to LPS plus IFN-γ by each Mϕ population appeared to be inversely related to IL-12 levels stimulated by LPS alone (Fig. 7). Thus, treatment with IFN-γ resulted in the normalization of IL-12 production among Mϕ from these strains. Strikingly, the levels of IL-12 produced by NOD Mϕ in

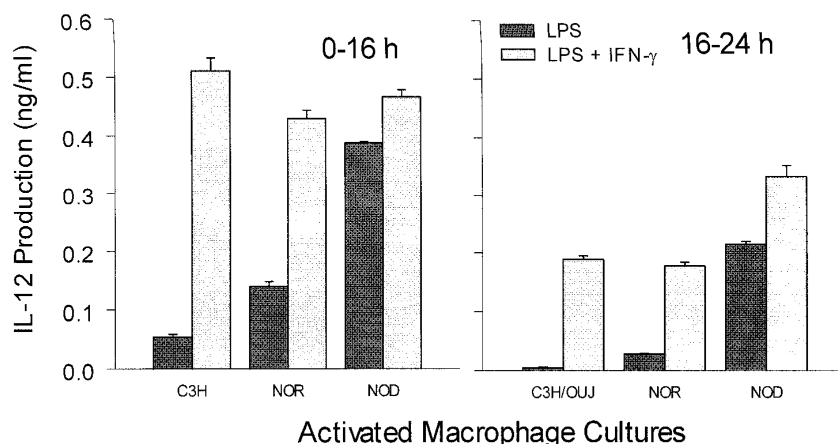

FIG. 7. IFN-γ normalizes IL-12 production among Mϕ from NOD and control strains. Two × 10^5 thioglycollate-elicited peritoneal Mϕ from NOD and 2 control strains (C3H/OuJ and NOR) were activated with 100 ng/ml LPS in the absence or presence of IFN-γ (10 ng/ml), and conditioned medium was collected from sequential cultures (as indicated) and assessed for IL-12 levels. Mean levels of IL-12 and SE of triplicate cultures from 1 experiment representative of at least 3 experiments per strain are shown.

response to LPS alone were similar to the levels produced by Mϕ from control strains after stimulation with both LPS and IFN-γ (Fig. 7). Further evaluation revealed that responsiveness to IFN-γ was stringently regulated, as demonstrated in additional experiments by the precise reciprocal correlation ($r^2 = 0.95$) between IL-12 levels induced by LPS alone and the enhancement of these levels by IFN-γ (Fig. 8). These data indicate that Mϕ from NOD mice are programmed to produce uniquely high levels of IL-12 in the absence of IFN-γ. This innate property has the potential to bias T-cell responses toward a Th1 phenotype, consistent with the development of organ-specific autoimmunity. Finally, it should be noted that the response of NOD Mϕ to IFN-γ does not itself appear to be aberrant because it does not diverge from the precise reciprocal relationship that characterizes the response of Mϕ from all control strains tested (Fig. 8). In addition, the extent of synergy between IFN-γ and LPS, vis-à-vis TNF-α induction, was virtually identical in C3H and NOD Mϕ, two strains that differ dramatically in response to IFN-γ modulation of IL-12 expression. In both cases, IFN-γ treatment produced a 4- to 4.5-fold increment in TNF-α production over the response to LPS alone (data not presented).

DISCUSSION

Abnormalities in Mϕ production of, or response to, cytokines have been associated with mouse models of both lupus and diabetes (9–15,24). However, it remains to be determined whether cytokine dysregulation in NOD Mϕ results from a genetic predisposition, as opposed to a modulation of cytokine expression by the disease process itself. This question prompted us to investigate whether Mϕ from young prediseased NOD mice express an intrinsically aberrant pattern of cytokine production

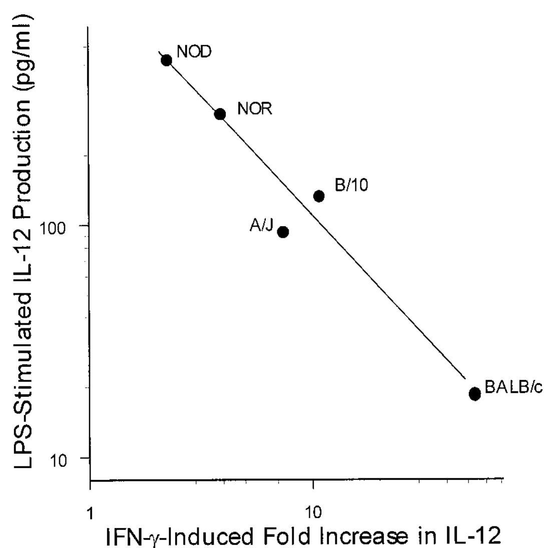

FIG. 8. IFN-γ enhancement of LPS-induced Mϕ IL-12 production demonstrates a strict inverse relation to the response to LPS alone. Two × 10^5 thioglycollate-elicited peritoneal Mϕ from NOD and 4 control strains (BALB/c, A/J, C57BL/10, and NOR) were activated with 100 ng/ml LPS in the absence or presence of IFN-γ (10 ng/ml), and conditioned medium was collected from cultures incubated for 16 h. IL-12 levels (ng/ml) induced by LPS alone were plotted relative to the enhancement (fold-increase) of these levels by IFN-γ. Each value represents the mean of triplicate values from 1 experiment representative of at least 3 experiments per strain. Linear regression analysis for all values produced a correlation coefficient (R^2) of 0.95.99.

and, additionally, if a specific pattern of cytokine dysregulation could be established that might provide insight into the origin or pathology of diabetes. The most striking findings to emerge from these studies are *1*) the precisely controlled balance of IL-10 and TNF-α characteristic of Mϕ from normal strains is not preserved in NOD mice, and *2*) Mϕ IL-12 production is dramatically elevated in this strain.

TNF-α and IL-1 have been reported to modulate the course of autoimmunity in murine models of lupus (13,29) and type 1 diabetes (14,30). This premise is perhaps most convincingly supported by the finding that administration of either cytokine after the onset of insulitis reduces the severity or blocks the development of diabetes (14,30). Although we and others have firmly established that Mϕ from lupus-prone mice (i.e., NZB/W F1 and MRL/++) express substantial intrinsic defects in production of TNF-α, IL-1, and IL-6 (but not IL-10 [13,24,31–34]), it has been unclear whether similar intrinsic defects are manifested in the NOD mouse. Several reports demonstrate that Mϕ from young prediseased NOD mice express levels of IL-1 and TNF-α that are roughly 3- to 5-fold less than levels of controls (9–12,14,15). However, the fact that the controls used in these studies (NON and SWR) produce high levels of TNF-α and IL-1 relative to other control strains (15) would skew the evaluation of the NOD response. We have found that levels of certain Mϕ cytokines (e.g., TNF-α and IL-10) vary by 20-fold or more among a broad panel of control strains (24,34). Here, we have clarified the extent of defective IL-1 and TNF-α expression in NOD Mϕ by comparing NOD cytokine production to that of the congenic NOR, as well as several more genetically diverse control strains. We found that although NOD Mϕ produced TNF-α and IL-1 levels that were within the range of the controls, these cytokine levels nevertheless seemed to reflect a disease-related trait because they were substantially less than that of the congenic NOR strain. This finding suggests that the extent to which a given cytokine modulates an immune pathway, or a defect in production of a specific cytokine contributes to a pathological process, may be dictated by multiple factors. These would include the cellular functions involved in the pathway, the absolute level of the cytokine in question, and the genetic background of a given strain.

A second type of defect was revealed by studies showing that although TNF-α levels vary substantially among the panel of strains tested, they are in fact rigidly determined in any normal strain by being tightly linked to and controlled by IL-10 expression; this results in a precise reciprocal relationship of the 2 cytokines (Fig. 3; 24). Thus, it is plausible that cytokine levels produced by any strain that deviate from this highly conserved regulation could contribute to immune dysfunction. Indeed, the fact that the balance of TNF-α and IL-10 in congenic NOR Mϕ is consistent with that of Mϕ from all other normal strains tested emphasizes the divergence of NOD Mϕ function. Interestingly, this cytokine relationship is also severely dysregulated in Mϕ from lupus-prone strains (24), revealing a conserved perturbation of the relationship of IL-10 and TNF-α in multigenic autoimmunity. That this perturbation may contribute to autoimmunity is suggested by the demonstration that administration, at the appropriate times, of TNF-α to NOD and NZB/W mice (1,13,14,35), or IL-10 to NOD (36) and anti–IL-10 to NZB/W F1 mice (37), blocks autoimmune disease. Whereas the mechanism by which TNF-α administration ameliorates autoimmune dis-

ease—and by which intrinsically deficient TNF-α levels might exacerbate disease—is not known, at least 2 possibilities can be offered. First, TNF-α, recognized as a proinflammatory mediator of leukocyte trafficking and migration, has also been shown to suppress T-cell effector function in a variety of systems (38). Thus, intrinsically reduced TNF-α levels could be conducive to enhanced autoreactivity. Second, the TNF-α receptor, homologous with Fas, can mediate apoptosis, and its most pronounced contribution to apoptosis is noted during excessive or chronic T-cell activation (39). A reduction in TNF-mediated apoptosis could thus lead to reduced peripheral tolerance in autoimmunity.

Reduced TNF-α and IL-1 production in NOD Mϕ may play a critical role at different stages of the development of diabetes in NOD mice. Elevated TNF-α levels, derived from recombinant cytokine administration, exacerbate disease in young prediseased mice (30), presumably by increasing the initial entry of inflammatory leukocytes into the pancreas and thus accelerating insulitis. Conversely, administration of TNF-α or IL-1 once insulitis has been initiated blocks the subsequent development of frank diabetes (14,40), perhaps by reducing the extent of T-cell activation, as discussed above. This scenario is further supported by findings from NOD transgenic mice in which TNF-α regulated by the rat insulin II promoter is expressed in the pancreas (43). Local TNF-α expression in these mice promotes the onset of insulitis, but also prevents subsequent β-cell destruction (43). These studies reveal that the same cytokine can have opposing effects on different stages of disease progression and provide additional evidence that aberrant expression of TNF-α may contribute to diabetes. IL-1 treatment also blocks the development of disease (14), perhaps by enhancing the development of regulatory T-cells (11), which may have a Th2 phenotype (44,45).

Whereas Mϕ from diabetes- and lupus-prone mice share a defect in TNF-α production, they are clearly distinguished functionally by the observation that Mϕ from NOD mice express substantially elevated IL-12 levels, whereas Mϕ from lupus-prone strains produce markedly reduced levels compared with all control strains tested (34). IL-12 is regarded as the key mediator of preferential Th1 development, and our results suggest that the genetically programmed aberrant levels of IL-12 produced by Mϕ from autoimmune-prone strains may have pathological significance not only in the initiation of autoimmunity, but in directing disease toward an organ-specific or humoral course. The balance of Th1/Th2 responses has been recognized as a critical factor in the development of immune pathology associated with *1*) models of spontaneous autoimmunity (e.g., type 1 diabetes [1,46]), *2*) experimentally induced organ-specific (47) and systemic (48) autoimmunity, and *3*) the unique susceptibility of BALB/c mice to infection by the parasite *Leishmania major* (49). However, it has not been resolved whether skewing toward an aberrant Th1 or Th2 response in these models of disease arises from intrinsic defects expressed within the activated lymphocytes or, alternatively, from aberrant modulation of lymphocyte function as a consequence of intrinsic defects in regulatory cells. Our findings give credence to the latter possibility by demonstrating that innate cytokine defects in Mϕ from autoimmune-prone mice are predictive of the Th response associated with the course of organ-specific or humoral autoimmunity. Thus, the elevated IL-12 levels readily produced by NOD Mϕ may contribute to the dominant and pathogenic Th1 response that mediates type 1 diabetes, whereas reduced IL-12 levels, noted in Mϕ from NZB/W and MRL mice (34), are consistent with the Th2-mediated B-cell responses characteristic of lupus. Moreover, the finding of elevated IL-12 production by NOD Mϕ in response to several stimuli suggests that this aspect of cytokine dysregulation could enhance autoreactive Th1 responses in diverse settings. First, the response to LPS and BCG suggests that microbial antigens that mimic self-proteins might trigger heightened IL-12 responses, a phenomenon recently associated with type 1 diabetes (50). Additionally, CD40-mediated responses to self (islet) antigens would also likely be skewed toward a Th1 pathway in NOD mice. That such events are actually mediated by Mϕ during the development of diabetes is supported by the recent finding that depletion of Mϕ in NOD mice leads to a shift from Th1 to Th2 skewing with a concomitant reduction in islet destruction (51). Moreover, administration of IL-12 is sufficient to initiate disease in previously healthy animals depleted of Mϕ (52). The association of intrinsic defects in Mϕ IL-12 production with polarized pathogenic Th responses in different disease-prone murine strains should provide insight into the cellular basis of immune deviation and may contribute to identification of specific genetic elements contributing to autoimmune diseases.

As with TNF-α, there is evidence that IL-12 plays a direct role in diabetes. Not only does IL-12 mRNA expression increase in NOD pancreatic islets concomitant with β-cell destruction (4), but, more compellingly, in vivo neutralization of IL-12 in NOD mice blocks disease (20,21). Conversely, IL-12 administration induces premature diabetes in NOD mice but not control (BALB/c) mice (19). However, a recent publication using IL-12 gene-deficient NOD mice appeared to suggest that IL-12 did not play an important role in the disease (53). A similar dichotomy between successful protection from disease by blocking cytokine function and unmodulated disease in cytokine-deficient NOD mice was reported in studies with IFN-γ (54,55). Whereas the mechanism underlying these differences is not clear, the evidence supporting a role for IL-12 (and IFN-γ) in disease in genetically unmanipulated NOD mice seems unquestionable. We suspect that these differences arise based on the time at which the cytokine pathway is blocked: at ~3 weeks of age with the cytokine antagonist compared with chronic and total cytokine depletion in the knockout mice. The latter process may interfere with the early establishment of important regulatory pathways and thus disrupt the normal pathway of disease (53). One mechanism might be the reported enhancement of IL-4–dependent Th2 induction by IL-12 (56).

It is likely that aberrant cytokine production is part of a complex pathway mediating autoimmune disease, in which multiple gene defects contribute in an epistatic manner. We interpret these findings, in sum, to indicate that although cytokine defects are not themselves capable of causing disease, they are sufficiently disruptive to appropriate immune function that they are rarely, if ever, seen in normal animals but appear as a common element in diverse models of autoimmunity. To our knowledge, dysregulation of the TNF-α/IL-10 axis as well as substantial reduction (lupus) or enhancement (diabetes) of IL-12 expression are the only intrinsic immune defects that have been conserved among several mouse models of spontaneous multigenic autoimmunity.

We anticipate that the study of intrinsic regulatory defects common to distinct models of multigenic autoimmunity will

provide mechanistic insights not only into end-stage pathology, but also into the fundamental immune dysregulation that triggers autoimmune disease. Ultimately, the contribution of any functional defect to the disease process can only be confirmed by identification of the specific genes within the loci that have been shown to protect from or predispose to autoimmunity.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant AI31418 and by a grant from the Juvenile Diabetes Foundation.

The authors thank Dr. Victor H. Van Cleave for rMu IL-12 and antibodies for the IL-12 ELISA; Genentech for rMu IFN-γ; Dr. Marilyn Kehry for CD40L; and Dr. Tim Heeren, Department of Epidemiology, Boston University Medical Center, for consulting on statistical analysis.

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