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CUTTING EDGE

Cutting Edge: Diabetes-Associated Quantitative Trait Locus, *Idd4*, Is Responsible for the *IL-12p40* Overexpression Defect in Nonobese Diabetic (NOD) Mice

Pedro B. Simpson, Monica S. Mistry, Richard A. Maki, Weidong Yang, David A. Schwarz, Eric B. Johnson, Francisco M. Lio, and David G. Alleva[1]

***APCs of the nonobese diabetic (NOD) mouse have a genetically programmed capacity to overexpress* IL-12p40*, a cytokine critical for development of pathogenic autoreactive Th1 cells. To determine whether a diabetes-associated NOD chromosomal locus (i.e.,* Idd*) was responsible for this defect, LPS-stimulated macrophages from several recombinant congenic inbred mice with* Idd *loci on a C57BL/6 background or with different combinations of NOD and CBA genomic segments were screened for* IL-12p40 *production. Only macrophages from the congenic strains containing the* Idd4 *locus showed* IL-12p40 *overproduction/expression. Moreover, analysis of* IL-12p40 *sequence polymorphisms demonstrated that the* Idd4 *intervals in these strains contained the* IL-12p40 *allele of the NOD, although further analysis is required to determine whether the* IL-12p40 *allele itself is responsible for its overexpression. Thus, the non-MHC-associated* Idd4 *locus appears responsible for* IL-12p40 *overexpression, which may be a predisposing factor for type 1 diabetes in NOD mice. The Journal of Immunology,* 2003, 171: 3333–3337.**

Multiple environmental and genetic factors interact to precipitate insulin-dependent diabetes mellitus (IDDM)[2], a spontaneous autoimmune disease in humans and in the nonobese diabetic (NOD) mouse (reviewed in Ref. 1). Although the genetic factors responsible for the different stages of IDDM have not yet been elucidated, quantitative trait loci analyses have been used to identify at least 18 major NOD chromosomal intervals, i.e., *Idd* loci, that contribute to the course of disease (reviewed in Refs. 2 and 3). Among them, the MHC H-2^{g7} haplotype within the *Idd1* locus is unique in that it is necessary (but not sufficient) for diabetes to develop. Although a few candidate genes have been nominated as potential aberrant genetic elements contained in non-MHC-linked *Idd* loci, their impact on the course of disease or on a disease-associated cellular process has been difficult to ascertain.

A major obstacle in identifying the IDDM-promoting genes within these non-MHC-linked *Idd* loci has been the lack of adequate disease-associated phenotypes with sufficient penetrance for tracking in recombinant congenic gene mapping studies (2). Most attempts have been made using complex traits such as disease incidence or cellular processes such as insulitis that likely require epistatic interactions among several *Idd* loci. However, we have identified and characterized a genetically-programmed, cell type-specific phenotype unique to the NOD strain that exists in the absence of the disease process (i.e., it is expressed in young male and female NOD mice well before disease symptoms (4, 5)), and thus is expected to show sufficient penetrance in recombinant congenic gene mapping studies. This defect is a 4- to 25-fold elevation in production of the *IL-12p40* cytokine subunit (but not of other cytokines) by activated macrophages (Mϕ) from the NOD strain compared with at least eight other non-diabetic control strains (4–6). This finding has recently been confirmed in dendritic cells by Tisch and coworkers (7), indicating that the defect is expressed by two key APCs of the innate immune system. The *IL-12p40* subunit complexes with either the p35 or the p19 subunits to form the functionally active IL-12p70 (8) or IL-23 (9) cytokines, respectively, that are required for development of Th1 responses and play a central role in the autoimmune process. Furthermore, elevated *IL-12p40* production has also been associated with individuals at high-risk for type 1 diabetes (10) in which unique sequence polymorphisms near the *IL-12p40* coding sequence have been identified in a large cohort of type 1 diabetic families (11).

Here, we determined whether a single *Idd* locus could be responsible for the *IL-12p40* expression defect in NOD Mϕ by screening several different recombinant congenic mouse strains carrying *Idd* loci on either C57BL/6 or CBA backgrounds. Our results show that the *Idd4* locus is responsible for the elevated expression of the *IL-12p40* gene in NOD mice.

Materials and Methods

Animals

Four- to 6-wk-old male mice of the following strains were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained for 1–4 wk after arrival under germfree conditions: A/J, BALB/c, C57BL/6, C57BL/10, C3H/

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[2] Abbreviations used in this paper: IDDM, precipitate insulin-dependent diabetes mellitus; NOD, nonobese diabetic; Mϕ, macrophage.

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OUJ, CBA, and NOD. The following CBA-NOD recombinant congenic strains that have unique sets of *Idd* loci (12) were a gift from Dr. E. Leiter (The Jackson Laboratory): NOcCB-1 (*Idd*1, 2, 4, 5, 6, 7, 8, 13, 14, 15, 16), CBcNO6 (*Idd*1, 2, 6, 8, 9, 11, 13, 15, 16), CBcNO7-C (*Idd*1, 6, 7, 9, 15, 16), and CBcNO7-D (*Idd*1, 6, 7, 9, 10, 13, 15, 16, 17). Breeding pairs of the following C57BL/6 mice congenic for *Idd* loci were also a gift from Dr. E. Leiter and bred in-house: B6.NODc17 (B6.NODIdd1), D17MIT21-D17MIT10, 19 cM interval; B6.NODc11 (B6.NOD*Idd4*), D11Mit20-D11Mit42, 52 cM interval; B6.NODc17/c11 (B6.NOD*Idd1*/*Idd4*), D17Mit21-D17Mit10, 19 cM interval, D11Mit20-D11Mit42, 52 cM interval; B6.NODc17/c1c (B6.NOD*Idd1*/*Idd5*), D17Mit21-D17Mit10, 19 cM interval, D1Mit3-*Bcl*II, 44 cM interval; B6.NODc17/c6 (B6.NOD*Idd1*/*Idd6*), D17MIT21-D17MIT10, 19 cM interval, D6MIT54-D6MIT14, 24 cM interval; B6.NODc17/c3 (B6.NOD*Idd1*/*Idd3*/*Idd10*), D17Mit21-D17Mit10, 19 cM interval, D3Mit132-Tshb, 43 cM interval; B6.NODc2 (B6.NOD*Idd13*), D2Mit17-D2Mit48, 32 cM interval (13).

Mϕ isolation, culturing, and cytokine production measurement

Thioglycollate-elicited peritoneal exudate Mϕ were obtained by peritoneal lavage and isolated by adherence to plastic as previously described (4). Adherent Mϕ (2×10^5) were stimulated with LPS (LPS; *Escherichia coli*: 0111:B4; Sigma-Aldrich, St. Louis, MO) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 0.5% HEPES (Cellgro, Herndon, VA), 5 μg/ml penicillin and 100 U/ml streptomycin (Life Technologies, Grand Island, NY), and 10% FBS (BioWhittaker, Walkersville, MD). The culture-conditioned medium was collected at 24 h at which time Mϕ were replenished with fresh stimuli and cultured for another 48 h before collection of conditioned medium (i.e., 24–72 h). Culture-conditioned medium was stored at −20°C for assessment of cytokine levels using sandwich ELISA (BD PharMingen, San Diego, CA).

RNase protection assay

RNA was extracted from LPS-stimulated adherent Mϕ (10^7) using a total RNA Isolation Kit (BD PharMingen) and quantified by UV spectrophotometry. Total RNA (5 μg) from each sample was used in an RNase protection assay (Riboquant mCK-2b; BD PharMingen) which was resolved using a 0.4 mm urea-polyacrylimide gel and visualized by autoradiography. Samples were quantified by densitometry using Image-Pro Plus software.

Sequencing IL-12p40 *cDNA polymorphisms*

Two T→C sequence polymorphisms exist in the cDNA sequence (accession number NM_008352) of the murine *IL-12p40* allele at nucleotide positions 506 and 880 down-stream from the transcription start site and distinguish the C57BL/6 and CBA (i.e., T) from the NOD (i.e., C) sequences (14). These polymorphisms were determined in several congenic strains via RT-PCR amplification of total RNA from LPS-stimulated Mϕ and sequencing of the PCR product using the following primers:_GACTTTCCTGAAGTGTGAAG CACC and GCTGACCTCCACCTGTGAGTTC which flank the polymorphism at 506 position, and GCAGCAGAATAAATATGAGAAC and CCTT TCCAACGTTGCATCCTAGG which flank the polymorphism at 880 position.

Results and Discussion

Overexpression of IL-12p40 *in the autoimmune-prone NOD strain: a comprehensive analysis*

We previously reported the *IL-12p40* overexpression defect in LPS-stimulated Mϕ from young (pre-disease) NOD mice (4, 5). Here, we determined whether one of the known *Idd* loci harbored the defect by screening several congenic strains that contained *Idd* loci on a C57BL/6 background. We first confirmed and extended our previous studies (4, 5) by performing a comprehensive analysis of *IL-12p40* levels produced by Mϕ from the C57BL/6 background strain relative to other disease-resistant strains (i.e., BALB/c, A/J, C57BL/10, C3H/OuJ) and to the autoimmune-prone NOD strain. Peritoneal Mϕ from young mice of all strains were activated with LPS, and conditioned medium was collected in two sequential intervals to quantitatively and qualitatively evaluate *IL-12p40* production. Cytokine levels for each strain were normalized to those of C57BL/6 Mϕ and the mean of these relative values from several experiments are reported (Fig. 1). LPS-stimulated Mϕ from the NOD strain produced 4- to 12-fold greater levels of *IL-12p40*

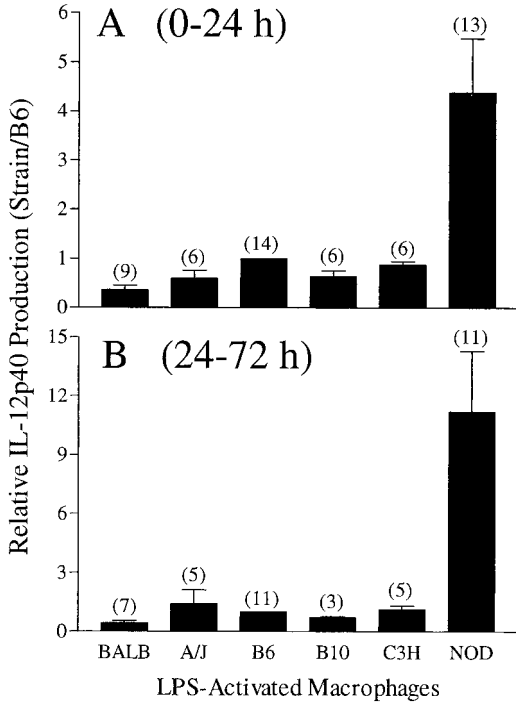

FIGURE 1. Elevated production of *IL-12p40* by Mϕ from the NOD strain. Thioglycollate-elicited peritoneal Mϕ (2×10^5) from five control strains (BALB/c, A/J, C57BL/6 (B6), C57BL/10 (B10), and C3H/OuJ) and the diabetes-prone NOD strain were activated with LPS (100 ng/ml) and incubated for 24 h (*A*) and from 24 to 72 h (*B*). (At the end of 24 h, conditioned medium was removed and cultures were replenished with fresh medium and LPS for an additional 48 h.) Total *IL-12p40* levels were measured using an ELISA. Shown are means ± SEM of several experiments per strain (N denoted in parentheses) of relative *IL-12p40* levels from each strain normalized to B6 levels which were given the value of 1. The mean ± SEM of B6 Mϕ *IL-12p40* levels from several experiments ($n = 8$) was 3.4 ± 0.9 ng/ml for 24-h cultures, and 0.6 ± 0.1 ng/ml for 24- to 72-h cultures.

than did Mϕ from the five normal strains tested during the first 24 h of culture (Fig. 1*A*). This difference increased to 10- to 25-fold greater *IL-12p40* levels in the NOD during the next 48 h of culture (i.e., 24–72 h; Fig. 1*B*). Differences in Mϕ cytokine production among all strains were not associated with a variation in viability or metabolic activity as measured by conversion of the redox reagent, Alamar Blue (data not shown). This dysregulation in cytokine expression appears specific to *IL-12p40* because NOD Mϕ levels of other cytokines are within the range of normal strains (4).

IL-12p40 *expression in Mϕ from B6.NODIdd congenic strains*

LPS-stimulated Mϕ from seven recombinant congenic strains on a C57BL/6 background containing single, double, or triple *Idd* loci were screened for the *IL-12p40* overproduction defect. Of the seven congenic strains screened, only those carrying the *Idd4* locus, i.e., B6.NOD*Idd4* and B6.NOD*Idd1*/*Idd4*, showed significantly elevated levels of *IL-12p40* production relative to the parental C57BL/6 strain (Fig. 2), demonstrating that the *Idd4* locus is the factor responsible for the *IL-12p40* overproduction defect. The 20% difference in *IL-12p40* levels between the B6.NOD*Idd4* and NOD strains is most likely due to elevated IL-10 levels (an inhibitory cytokine) in the C57BL/6 background: C57BL/6 and the B6.NOD*Idd4* congenic Mϕ produce 4-fold more IL-10 than NOD Mϕ (Ref. 4

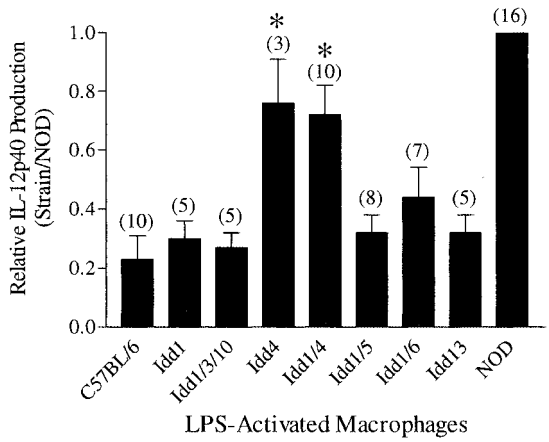

FIGURE 2. Elevated production of *IL-12p40* by Mϕ from the B6.NOD*Idd4* and B6.NOD*Idd1*/*Idd4* congenic strains. Thioglycollate-elicited peritoneal Mϕ (2×10^5) from seven B6.NOD*Idd*-congenic strains containing single (*Idd1, Idd4, Idd13*), double (*Idd1/4, Idd1/5, Idd1/6, Idd1/7*) or triple (*Idd1/3/10*) *Idd* loci on a C57BL/6 background, and from the normal C57BL/6 and diabetes-prone NOD control strains were activated with LPS (100 ng/ml) and incubated for 24 h. Conditioned medium was collected and *IL-12p40* levels were measured by ELISA. Shown are means ± SEM of several experiments per strain (N denoted in parentheses) of relative *IL-12p40* levels from each strain normalized to those of the NOD which were given the value of 1. *, Significantly ($p < 0.05$) different from C57BL/6 mean values. The mean ± SEM of NOD Mϕ *IL-12p40* levels from several experiments ($n = 11$) was 14.8 ± 2.3 ng/ml for 24-h cultures.

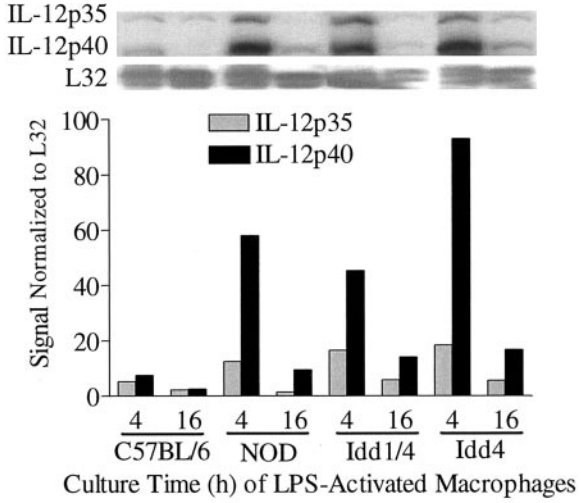

FIGURE 3. Elevated expression of *IL-12p40* mRNA by the B6.NOD*Idd4* and B6.NOD*Idd1*/*Idd4* congenic strains. Thioglycollate-elicited peritoneal Mϕ (10^7) from C57BL/6, NOD, B6.NOD *Idd4,* and B6.NOD *Idd1*/*Idd4* strains were activated with LPS (100 ng/ml) for 4 and 16 h. Total RNA was extracted and mRNA levels of *IL-12p40* and IL-12p35 subunits were assessed in an RNase protection assay. Samples were separated on a sequencing gel and visualized by autoradiography. The intensities of each *IL-12p40* and p35 mRNA band were normalized to the loading control L32 mRNA band via densitometry analysis using the ImagePro software. Results represent one of at least three experiments that had similar results.

and data not shown). Moreover, Ab-mediated neutralization of IL-10 restored *IL-12p40* levels of the *Idd4* congenic strains (but not C57BL/6) to those of the NOD strain (data not shown). As a control for the *Idd4*-specific effects on the *IL-12p40* gene, TNF-α levels were similar among all strains (i.e., less than 2-fold differences; data not shown), which is consistent with our previous observations of C57BL/6 and NOD Mϕ TNF-α levels (4).

B6.NODIdd4 congenic and NOD Mϕ express similar levels of IL-12p40 *mRNA*

We confirmed that the *IL-12p40* overproduction defect was associated with the *Idd4* locus by comparing mRNA levels expressed among the C57BL/6, B6.NOD*Idd4*, B6.NOD*Idd1*/*Idd4*, and NOD strains (Fig. 3). Maximal expression of *IL-12p40* mRNA occurred at 4 h in Mϕ from all four strains; Mϕ from the NOD, B6.NOD*Idd4*, and B6.NOD*Idd1*/*Idd4* strains expressed similar levels, each of which were 6- to 12-fold greater than those of the C57BL/6 at 4 h. Maximum IL-12p35 mRNA expression among all strains occurred at 4 h at which time differences among strains were roughly 2- to 3-fold. Moreover, a distinguishing qualitative difference is that Mϕ expression of *IL-12p40* mRNA was substantially greater than that of IL-12p35 mRNA only in the NOD, B6.NOD*Idd4*, and B6.NOD*Idd1*/*Idd4* strains, whereas mRNA levels of these two subunits were similar in the C57BL/6 (Fig. 3) and in other control strains as well (4, 5). Note that we have previously shown that the *IL-12p40* expression defect by NOD Mϕ leads to overproduction of the functional heterodimer, IL-12p70 (4, 5). These results further support the association of the *IL-12p40* defect with the *Idd4* locus.

The Idd4 genomic interval includes the NOD IL-12p40 *allele*

Although the *Idd4* locus (15) and the *IL-12p40* gene, i.e., *IL-12b* (16), map to murine chromosome 11, it is unclear whether the *IL-12b* gene, which maps to the 19 cM position, is contained within the *Idd4* interval of the B6.NOD*Idd4* congenic strain, which starts around the 16–20 cM position (Ref. 13 and see Table I). To address this, we made use of two different single-nucleotide polymorphisms located within the *IL-12p40* cDNA that distinguish the C57BL/6 (and the CBA) from the NOD sequence (i.e., T→C (B6→NOD) at both 506 and 880 nucleotide positions, see *Materials and Methods* and Ref. 14). Sequencing of the two single-nucleotide polymorphisms in cDNA from all strains studied in this report showed that strains containing the *Idd4* interval (and therefore the *IL-12p40* defect) also contained the NOD *IL-12b* allele, and that those strains that did not contain the *Idd4* locus contained the B6 (and CBA, see below) *IL-12b* allele (Table I).

To confirm the association of the *IL-12p40* functional defect with the *Idd4* locus, *IL-12p40* production was assessed in four recombinant congenic strains containing roughly 50% NOD and 50% CBA genomes but varied in the combinations of *Idd* loci (12). Only one recombinant congenic strain, NOcCB-1, showed elevated-production (Fig. 4*A*) and elevated-expression (Fig. 4*C*) of *IL-12p40* relative to the CBA control strain. As previously noted for the NOD, B6.NOD*Idd4*, and B6.NOD*Idd1*/*Idd4* strains (see Fig. 3), expression levels of *IL-12p40* mRNA were substantially greater than those of IL-12p35 mRNA only in the NOD and NOcCB-1 strains (Fig. 4*C*). TNF-α levels were similar among all strains tested (Fig. 4*B*), confirming that the underlying molecular aberrancy does not effect the general Mϕ activation pathway, but rather is focused on *IL-12p40* expression in the NOcCB-1 and NOD strains. The NOcCB-1 strain was the only one of the four congenic strains to contain the entire *Idd4* locus (see *Materials and*

Table I. *Genotypic characterization of the* IL-12b *allele and* Idd4 *intervals in recombinant congenic murine strains*

Strain[a]	IL-12p40 Allele[b]		*IL-12p40* Expression Defect	Idd4 Locus[c]	
	506 nt	880 nt		Interval (cM)	Markers
C57BL/6	T	T	−	None	None
B6.NOD.Idd1	T	T	−	None	None
B6.NOD.Idd1/3/10	T	T	−	None	None
B6.NOD.Idd4	C	C	+	20–72	D11Mit20^{-} D11mit42
B6.NOD.Idd1/Idd4	C	C	+	20–72	D11Mit20^{-} D11mit42
B6.NOD.Idd1/Idd5	T	T	−	None	None
B6.NOD.Idd1/Idd6	T	T	−	None	None
B6.NOD.Idd13	T	T	−	None	None
CBA	T	T	−	None	None
CBc.NO7C	T	T	−	40–48	D11Acrb^{-} D11Mit36
CBc.NO6	T	T	−	40–70	D11Acrb^{-} D11Mit214
NOcCB-1	C	C	+	17–40	D11Mit84^{-} D11Acrb
NOD	C	C	+	n.a.	n.a.
DBA/2	C[d]	C[d]	−[d]	n.a.	n.a.

[a] Recombinant congenic strains with Idd4 loci on a C57BL/6 background (13) or with roughly 50% CBA and 50% NOD genomes with different combinations of Idd loci (12).
[b] Polymorphism in the IL-12p40 allele that distinguishes the NOD from the C57BL/6 and CBA alleles (14).
[c] Idd4 intervals on murine chromosome 11 defined by different microsatellite markers (12, 13).
[d] Refs. 6 and 14.

Methods and Ref. 12 for a detailed comparison of strains) and the NOD *IL-12b* allele (Table I). These results confirm that the *IL-12p40* expression defect is caused by the *Idd4* locus, which encompasses the NOD *IL-12b* allele.

It is unclear whether the NOD *IL-12b* allele is responsible for the overexpression of *IL-12p40* because we have previously reported (6) that the DBA/2, NZB, NZW, and NZB/W strains, which have the NOD *IL-12b* allele (14), do not overexpress

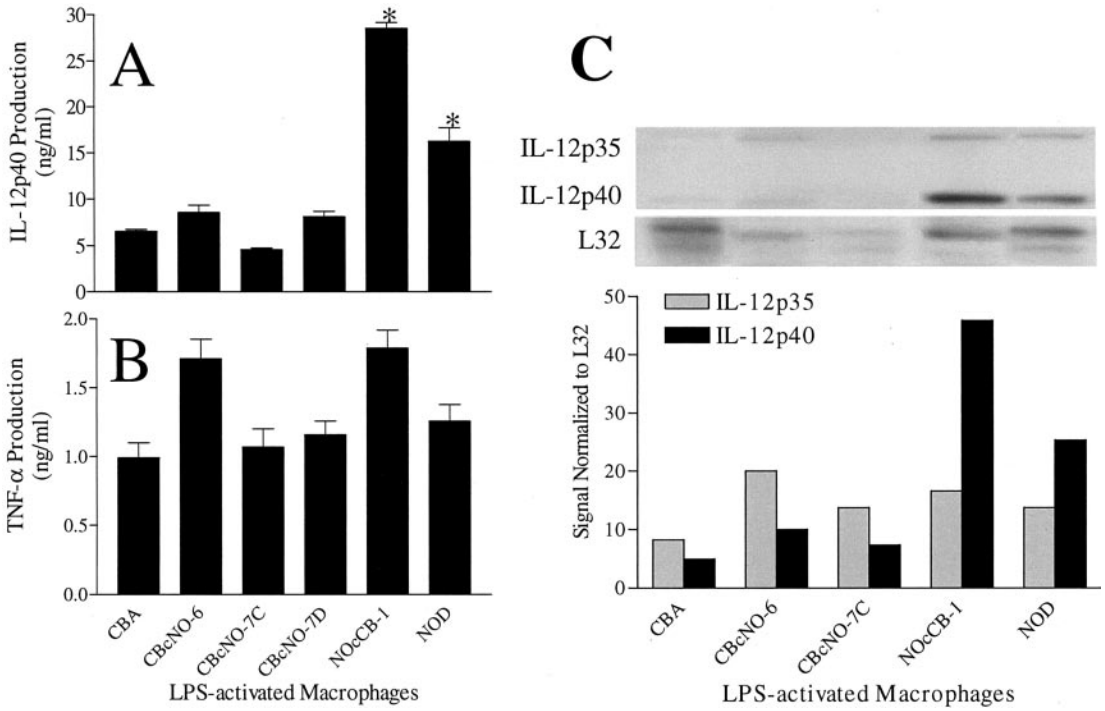

FIGURE 4. Elevated production of *IL-12p40* by a CBA-NOD recombinant congenic strain containing the *Idd4* locus. Thioglycollate-elicited peritoneal Mϕ (2×10^5) from four CBA-NOD recombinant congenic strains (CBcNO6, CBcNO7-C, CBcNO7-D, and NOcCB-1), each containing a different set of *Idd* loci, were activated with LPS (100 ng/ml) and incubated for 24 h. Conditioned medium was collected and *IL-12p40* (*A*) and TNF-α (*B*) levels were measured by ELISA. Shown are mean ± SEM values of triplicate cultures from one of two experiments that had similar results. *C,* mRNA levels of 4-h LPS (100 ng/ml)-activated Mϕ (10^7) from each strain that were assessed using the RNase protection assay. The intensities of each *IL-12p40* and p35 mRNA band were normalized to the loading control L32 mRNA band via densitometry analysis using the ImagePro software.

IL-12p40 (see Table I). Further, we observed no sequence polymorphisms in either the *IL-12p40* promoter region (i.e., 800 bases upstream from the transcriptional start site) or the 3′ untranslated region (i.e., 750 bases down-stream from the TAG translational stop codon) that were unique to the NOD strain relative to five normal strains (i.e., BALB/c, C57BL/6, C57BL/10, C3H/Ouj, A/J; data not shown). These observations suggest that the underlying genetic element responsible for the *IL-12p40* overexpression defect lies outside of the *IL-12b* gene but within the *Idd4* locus. Interestingly, Delovitch and coworkers (15) have mapped the T lymphocyte hypoproliferative phenotype to the *Idd4* locus using the NOD background congenic for a B6 resistant locus which spans a 5.2 cM interval at positions 43.8 to 49 cM (i.e., markers D11Nds1 and D11Mit38/325, respectively). However, it is unlikely that this interval confers *IL-12p40* overexpression because the CBcNO-6 and CBcNO-7 strains contain this 5.2 cM NOD interval (see Table I), but produced normal levels of *IL-12p40* (see Fig. 4). Therefore, the aberrant genes causing the *IL-12p40* expression defect are most likely within other subintervals of the *Idd4* interval, which could be further addressed by creating congenic strains containing a finer resolution of the interval. In addition, a comparative microarray mRNA expression analysis of LPS-stimulated Mϕ from *Idd* congenic and normal strains may reveal the genes responsible for the *IL-12p40* expression defect. As the mouse genome sequence is near completion, this genomic information should be useful in uncovering the genetic aberrancies within disease-associated loci of autoimmune-prone strains.

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