

# Discovery of Gene Networks Regulating Cytokine-Induced Dysfunction and Apoptosis in Insulin-Producing INS-1 Cells

Burak Kutlu,<sup>1</sup> Alessandra K. Cardozo,<sup>1</sup> Martine I. Darville,<sup>1</sup> Mogens Kruhøffer,<sup>2</sup> Nils Magnusson,<sup>2</sup> Torben Ørntoft,<sup>2</sup> and Décio L. Eizirik<sup>1</sup>

Locally released cytokines contribute to  $\beta$ -cell dysfunction and apoptosis in type 1 diabetes. In vitro exposure of insulin-producing INS-1E cells to the cytokines interleukin (IL)-1 $\beta$  + interferon (IFN)- $\gamma$  leads to a significant increase in apoptosis. To characterize the genetic networks implicated in  $\beta$ -cell dysfunction and apoptosis and its dependence on nitric oxide (NO) production, we performed a time-course microarray analysis of cytokine-induced genes in insulin-producing INS-1E cells. INS-1E cells were exposed in duplicate to IL-1 $\beta$  + IFN- $\gamma$  for six different time points (1, 2, 4, 8, 12, and 24 h) with or without the inducible NO synthase (iNOS) blocker N<sup>G</sup>-monomethyl-L-arginine (NMA). The microarray analysis identified 698 genes as cytokine modified ( $\geq 2.5$ -fold change compared with control) in at least one time point. Based on their temporal pattern of variation, the cytokine-regulated genes were classified into 15 clusters by the k-means method. These genes were further classified into 14 different groups according to their putative function. Changes in the expression of genes related to metabolism, signal transduction, and transcription factors at all time points studied indicate  $\beta$ -cell attempts to adapt to the effects of continuous cytokine exposure. Notably, several apoptosis-related genes were modified at early time points (2–4 h) preceding iNOS expression. On the other hand, 46% of the genes modified by cytokines after 8–24 h were NO dependent, indicating the important role of this radical for the late effects of cytokines. The present results increase by more than twofold the number of known cytokine-modified genes in insulin-producing cells and yield comprehensive information on the role of

NO for these modifications in gene expression. These data provide novel and detailed insights into the gene networks activated in  $\beta$ -cells facing a prolonged immune assault. *Diabetes* 52:2701–2719, 2003

**T**he  $\beta$ -cell dysfunction and death in type 1 diabetes and following islet transplantation is the result of direct contact with activated macrophages and T-cells, and/or exposure to soluble mediators secreted by these cells, such as cytokines, oxygen free radicals, and nitric oxide (NO) (1). In vitro,  $\beta$ -cell exposure to interleukin (IL)-1 $\beta$  alone induces functional impairment, whereas exposure to IL-1 $\beta$  in combination with interferon (IFN)- $\gamma$  and/or tumor necrosis factor- $\alpha$  induces  $\beta$ -cell death by apoptosis in rodent and human islets of Langerhans after a period of 4–9 days (1).

Cytokines modify the expression of several genes in the  $\beta$ -cell (1). An indirect proapoptotic effect of cytokines is the upregulation of the Fas receptor in rodent and human  $\beta$ -cells, increasing the susceptibility of these cells to apoptosis mediated by the Fas ligand expressed on islet-infiltrating macrophages and T-cells (2). IL-1 $\beta$  and IFN- $\gamma$  also play a role in the inflammatory destruction of islet grafts immediately after transplantation (1,3,4), a process that hampers the success of islet transplantation in patients with type 1 diabetes. This inflammatory environment induces expression of Fas in the transplanted  $\beta$ -cells (5) and chemokines, such as the macrophage chemoattractant protein-1 (MCP-1) (6,7), fractalkine, interferon inducible protein-10 (IP-10), and macrophage inflammatory protein-3 $\alpha$  (MIP-3 $\alpha$ ) (8,9), contributing to mononuclear cell homing (9). There is increasing evidence that apoptosis is the main mode of  $\beta$ -cell death in the development of type 1 diabetes and after islet transplantation (1). Commitment to apoptosis, a highly regulated process, is affected by extracellular signals, intracellular ATP levels, phosphorylation cascades, and expression of diverse pro- and antiapoptotic genes (1), which remain to be identified.

We have described by microarray analysis more than 200 genes and expressed sequence tags (ESTs) that are up- or downregulated by a 6- or 24-h exposure of rat  $\beta$ -cells to IL-1 $\beta$  and/or IFN- $\gamma$  (8,10). Cytokines induce stress-response genes that are either protective or deleterious for  $\beta$ -cell survival, whereas several genes related to differentiated  $\beta$ -cell functions are downregulated. Several cytokine-induced genes are potentially regulated by the

From the <sup>1</sup>Laboratory of Experimental Medicine, Université Libre de Bruxelles, Brussels, Belgium; and the <sup>2</sup>Molecular Diagnostic Laboratory, Department of Clinical Biochemistry, Aarhus University Hospital, Skejby, Denmark.

Address correspondence and reprint requests to Décio L. Eizirik, Laboratory of Experimental Medicine CP 618, Université Libre de Bruxelles, Route de Lennik, 808, B-1070, Brussels, Belgium. E-mail: deizirik@ulb.ac.be.

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AS, argininosuccinate synthetase; EST, expressed sequence tag; GADD, growth arrest and DNA damage; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GTP, guanosine triphosphate; GTPCH, GTP cyclohydrolase I; HMG, 3-hydroxy 3-methylglutaryl; hsp, heat shock protein; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; IP-10, interferon inducible protein-10; MCP, macrophage chemoattractant protein, MHC, major histocompatibility complex; MIP-3 $\alpha$ , macrophage inflammatory protein-3 $\alpha$ ; MnSOD, manganese superoxide dismutase; NF, nuclear factor; NMA, N<sup>G</sup>-monomethyl-L-arginine; Pdx-1, pancreatic duodenal homeobox factor-1; PI, propidium iodide; SERCA2b, sarco(endo)plasmic reticulum Ca<sup>2+</sup> ATPase type 2 b; VDCC, voltage-dependent anion channel.

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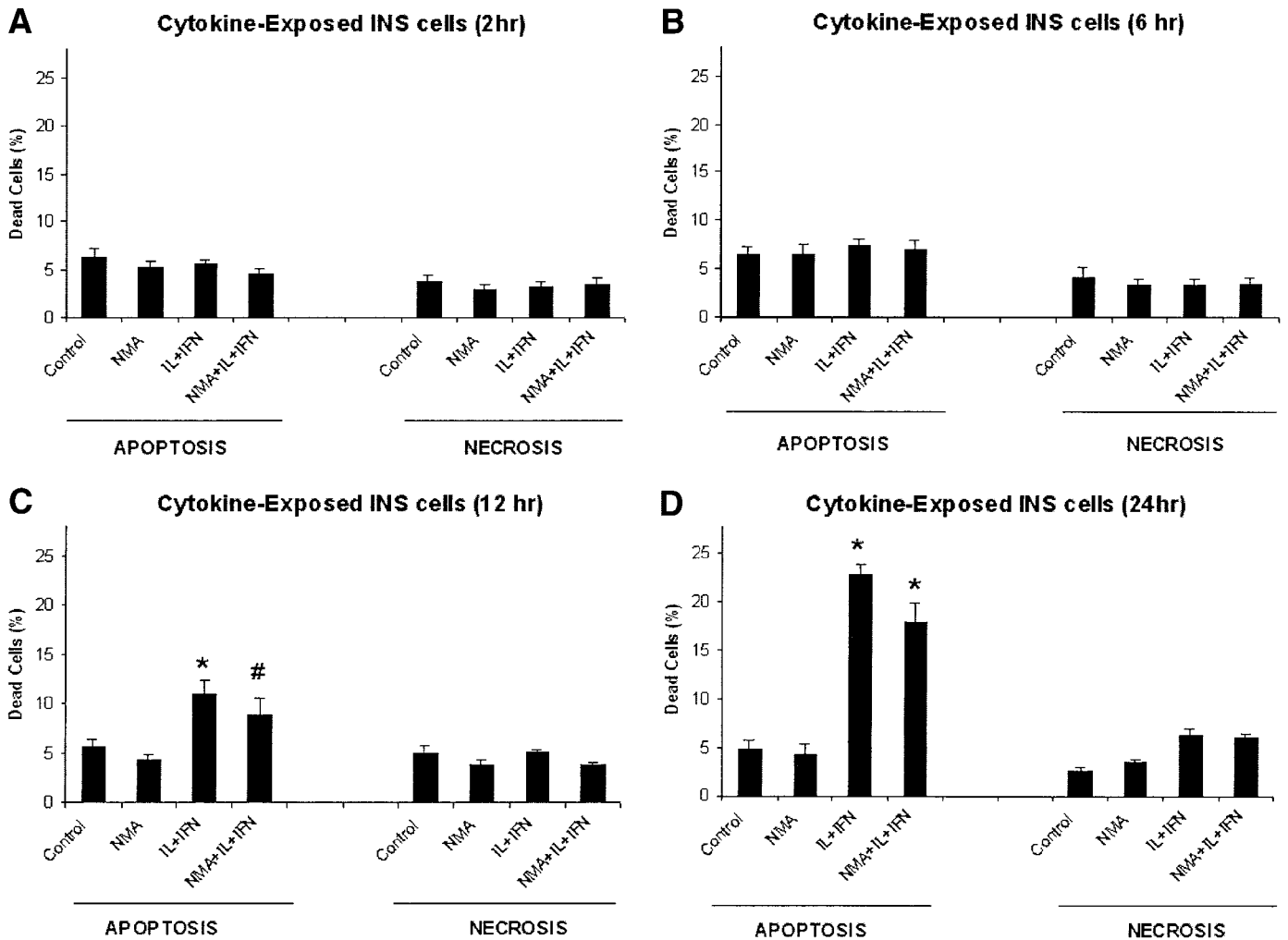
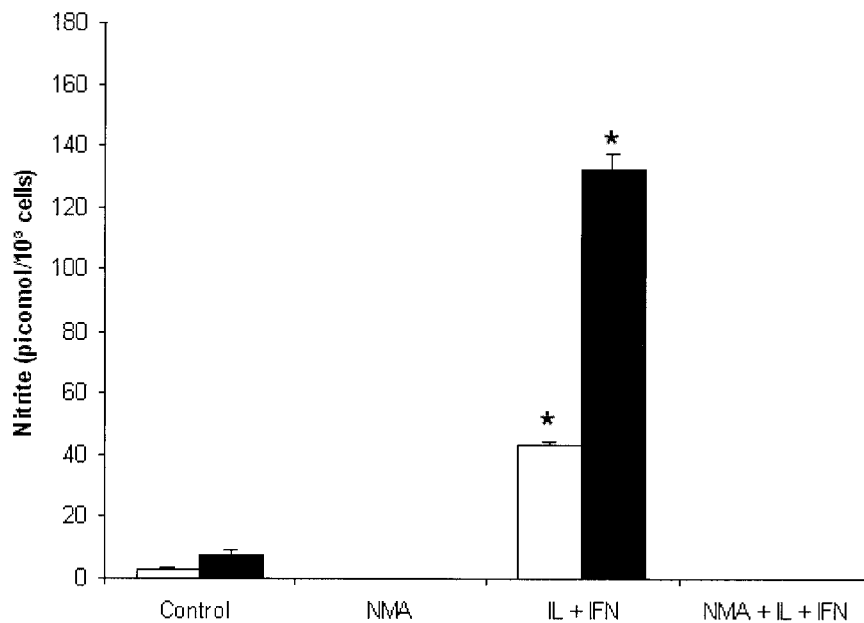


FIG. 1. Cytokine-induced death in INS-1E cells. INS-1E cells were treated for 2 h (A), 6 h (B), 12 h (C), or 24 h (D) with NMA, IL-1 $\beta$  + IFN- $\gamma$ , or NMA + IL-1 $\beta$  + IFN- $\gamma$  or left untreated (control). Cell viability was determined with the dyes HO 342 and PI. Apoptosis or necrosis is expressed as a percentage of the total number of cells counted. Results are means  $\pm$  SE of four experiments.  $^{\#}P < 0.05$ ;  $^*P < 0.001$  vs. control; paired *t* test.

transcription factor nuclear factor (NF)- $\kappa$ B (10). Inhibition of cytokine-induced NF- $\kappa$ B activation by adenovirus-mediated expression of a NF- $\kappa$ B super-repressor (I $\kappa$ B<sup>(SA)2</sup>) significantly improves  $\beta$ -cell survival, mainly through inhibition of apoptosis (10–12). The microarray experiments described above were performed at two time points (6 and 24 h) after cytokine exposure and did not allow discrimination between early and late effects of cytokines on gene expression. Moreover, they did not provide a detailed information on the pattern of NO-dependent genes. Therefore, we performed a detailed time-course microarray analysis to detect transient regulation of gene expression by cytokines in the presence or absence of the inducible NO synthase (iNOS) blocker *N*<sup>G</sup>-methyl-L-arginine (NMA). These data allowed classification of cytokine-induced genes in clusters based on their function and on their temporal profile of expression.

Cluster analysis of microarray data allows an integrated understanding of biological processes and provides indication of the function of novel genes coexpressed with genes of known function (13). It is empirically stated that clustering analysis of microarray studies requires at least five time point measurements. The large number of cells

required for a detailed time-course microarray analysis (around  $6 \times 10^7$  cells) makes it impossible to use purified primary  $\beta$ -cells in these experiments. Therefore, we chose to perform the present experiments using the well-differentiated insulin-producing cell line INS-1E (14). Cells were treated in parallel over a period of 1–24 h (six time points) with the cytokines IL-1 $\beta$  + IFN- $\gamma$  and/or NMA. The time points and combination of cytokines were chosen based on detailed time-course analysis for induction of apoptosis. Microarray analysis was performed using the Affymetrix system, as described in previous publications by our group (8,10). With this approach, we detected 698 differentially regulated mRNAs in response to cytokines, of which >60% are novel. Forty-six percent of the cytokine-regulated genes were NO dependent, highlighting the importance of NO in late regulation of gene expression. By using k-means cluster analysis (15), the detected genes were assigned to 15 distinct temporal profiles. The present set of microarray data opens several new lines of research, which are discussed below, and provides a detailed and comprehensive resource for scientists interested in the mechanisms of  $\beta$ -cell dysfunction and death in type 1 diabetes.



**FIG. 2.** Nitrite production by INS-1E cells exposed to IL-1 $\beta$  + IFN- $\gamma$  and/or NMA for 12 h ( $\square$ ) or 24 h ( $\blacksquare$ ). Results are the means  $\pm$  SE of four experiments. \* $P$  < 0.001 vs. control; paired  $t$  test.

## RESEARCH DESIGN AND METHODS

**Cell culture and nitrite measurement.** Insulin-producing INS-1E cells, a kind gift from Prof. C. Wollheim (Center Medical Universitaire, Geneva, Switzerland), were cultured in RPMI 1640 (with Glutamax-1) supplemented with 10 mmol/l HEPES, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 1 mmol/l sodium pyruvate, and 50  $\mu$ mol/l 2-mercaptoethanol (14,16). For the time-course microarray analysis, INS-1E cells ( $1.3 \times 10^6$  cells/condition) were exposed for 1, 2, 4, 8, 12, and 24 h to recombinant human IL-1 $\beta$  (10 units/ml; kindly provided by Dr. C.W. Reynolds, National Cancer Institute, Bethesda, MD) plus recombinant murine IFN- $\gamma$  (100 units/ml; Invitrogen, Baesley, Scotland), with or without 1.0 mmol/l of the iNOS inhibitor, NMA. The time points and the concentrations of cytokines and NMA used in the time-course analysis were selected based on our previous dose-response and time course studies on INS-1E cells, using cell survival,

nitrite production and expression of iNOS, MCP-1, and insulin mRNAs as end points (Fig. 1) (data not shown). The time-course microarray experiments were performed on two separate occasions (i.e., two independent experiments) using a total of  $6.2 \times 10^7$  INS-1E cells from passages 64 and 65. Culture media were collected at different time points for nitrite determination (nitrite is a stable product of NO oxidation), which was performed spectrophotometrically at a 546-nm wavelength after colored reaction with the Griess reagent (17).

**Assessment of INS-1E cell viability.** The percentage of viable, apoptotic, and necrotic INS-1E cells was determined after 2, 6, 12, and 24 h exposure to IL-1 $\beta$  + IFN- $\gamma$  with or without NMA. For this purpose, INS-1E cells were incubated for 15 min with propidium iodide (PI) (10  $\mu$ g/ml) and Hoechst HO 342 (20  $\mu$ g/ml) (18). This method has been successfully used to evaluate apoptosis/necrosis in human, rat, and mouse  $\beta$ -cells (18–21). A minimum of 500 cells was counted in each experimental condition.

TABLE 1

Functional clustering of differentially expressed genes in response to IL-1 $\beta$  + IFN- $\gamma$

	Percentage of the total	NO independent	NO dependent
	100 (698 genes)	53.5	46.5
1.0 metabolism (total)	19.6	41.1	58.9
1.1 carbohydrates	2.9	47.4	52.6
1.2 arginine metabolism and NO formation	0.9	66.7	33.3
1.3 amino acids (other than arginine)	2.8	33.3	66.7
1.4 lipids	5.4	42.9	57.1
1.5 ATP production and processing	1.2	12.5	87.5
1.6 miscellaneous	3.2	42.9	57.1
2.0 protein synthesis, modification, and secretion	6.4	52.4	47.6
3.0 ionic channels, ion transporters, and related	4.4	44.8	55.2
4.0 hormones and growth factors	9.5	51.6	48.4
5.0 cytokines, chemokines, and related receptors	2.4	87.5	12.5
6.0 cytokine processing and signal transduction	15.3	64.0	36.0
7.0 MHC and related genes	4.1	100.0	0
8.0 cell adhesion, cytoskeleton, and related genes	5.5	61.1	38.9
9.0 transcription factors and related genes	13.0	51.8	48.2
10.0 RNA synthesis	1.2	37.5	62.5
11.0 cell cycle and related genes	4.3	28.6	71.4
12.0 defense/repair	5.4	48.6	51.4
13.0 apoptosis/endoplasmic reticulum stress related	3.4	50.0	50.0
14.0 miscellaneous	8.7	50.9	49.1

Data are %. Cytokine-induced differences in gene expression were considered present when the mean fold change of the duplicates was  $\geq 2.5$  and both individual fold change values were  $\geq 2.0$  in at least one time point studied. Cytokine-modified genes were considered as NO dependent if their expression was increased/decreased  $>50\%$  by NMA in at least one time point and increased/decreased  $>30\%$  at a second time point.

TABLE 2  
List of selected genes modified by cytokines during a 24-h follow-up period

Functional group/GAN	Gene name	1 h		2 h		4 h		8 h		12 h		24 h	
		cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk
1.0 metabolism													
1.1 carbohydrates													
NO dependent													
X53588	Glucokinase	-1.1	-1.1	-1.3	-1.4	-2.3	-1.7	-2.8	-1.4	-3.7	-0.5	-3.1	-1.2
M13979	GLUT1	-1.1	0.1	0.0	-1.2	-2.3	-2.9	-2.4	-2.5	2.6	-1.6	3.3	0.0
U07181	Lactate dehydrogenase A	1.2	-1.5	-1.6	-1.6	-2.1	-2.3	-3.9	-2.0	-2.8	-1.6	-2.2	-3.1
X05684	L-type pyruvate kinase	0.0	-1.4	-1.5	-1.5	-1.6	-1.9	-2.6	-3.9	-4.1	-2.5	3.5	1.9
NO independent													
D87240	6-phosphofructo-2-kinase	0.7	1.9	2.3	4.0	4.3	3.1	8.4	7.4	4.6	3.4	-1.3	0.2
U20643	Aldolase A	-1.2	-0.1	15.3	25.4	-1.5	-1.2	-0.7	0.5	-0.3	1.3	1.2	2.2
I25387	Phosphofructokinase C	1.1	1.2	1.3	1.6	3.3	3.6	4.8	5.3	5.5	5.9	3.3	4.6
U32314	Pyruvate carboxylase	-1.2	-1.1	-0.1	-1.1	-1.2	1.2	-5.3	-2.3	-1.5	-1.7	0.0	0.2
U10357	Pyruvate dehydrogenase kinase 2 p45	0.3	-2.7	-1.1	0.0	0.2	-2.8	3.6	4.6	4.8	3.4	3.5	4.0
1.2 arginine and NO formation													
NO dependent													
AA957917	Cationic amino acid transporter-1	0.7	0.4	1.2	-1.5	0.1	-1.3	1.4	0.2	4.8	1.5	4.4	-1.2
NO independent													
X12459	Argininosuccinate synthetase	-1.1	-1.2	0.1	-0.3	1.5	0.2	12.4	15.9	34.8	41.7	12.4	24.9
E03424	GTP cyclohydrolase I	1.3	1.2	1.6	1.6	1.9	2.0	4.1	4.4	3.9	4.5	0.0	3.9
D44591	iNOS*	-0.2	1.2	0.0	1.9	33.6	31.5	194.5	190.0	96.1	117.1	18.4	38.6
M80804	L-type neutral amino acid transporter	0.0	0.0	2.6	2.7	-1.5	-1.5	-2.0	-1.9	-2.2	-2.8	-3.4	-2.0
1.3 amino acids													
NO dependent													
J02827	α-Ketoacid dehydrogenase E1-α subunit	2.1	2.0	-1.8	0.0	-3.2	-7.2	-8.7	-7.3	-6.0	-10.3	13.0	-2.3
A1176504	Glutaminase	-1.1	-1.0	-0.1	0.0	-3.0	-3.0	-7.9	-8.1	-10.8	-6.6	-16.9	-4.4
A1102838	Isovaleryl-CoA dehydrogenase	-1.3	-1.1	-1.2	-1.3	-0.1	2.0	-10.4	-4.9	-3.4	-8.7	-11.0	-2.9
AA891738	Sulfite oxidase	-1.1	-0.1	-1.1	0.0	-1.6	-1.7	-4.6	-2.6	-5.8	-2.4	-3.8	-1.6
NO independent													
A1233216	Glutamate dehydrogenase	1.2	0.1	-1.3	-1.2	-1.1	-1.1	-1.9	-1.9	-2.9	-2.8	-1.9	-1.6
A1008131	S-adenosylmethionine decarboxylase	1.1	-0.2	1.1	0.0	-1.7	-2.4	-1.9	-1.5	-2.6	-1.5	-1.5	-1.9
1.4 lipids													
NO dependent													
X13527	Acyl carrier protein domain of fatty acid synthetase	-0.4	-0.2	6.3	10.1	-2.7	-2.2	-3.4	-1.8	-1.8	0.0	-5.7	0.0
L27075	ATP-cytrate lyase	0.2	1.1	6.0	16.5	-2.3	-2.1	-2.4	-2.0	-1.7	-1.4	-7.0	-0.2
J05470	Carnitine palmitoyltransferase II	-1.1	0.0	0.1	0.1	-1.8	-2.2	-2.8	-1.9	-2.1	-1.2	-2.6	-1.1
M76767	Fatty acid synthase	1.0	0.1	-1.4	-1.8	-0.2	-1.3	-1.4	-1.3	-2.4	-1.4	-2.9	-1.3
M29249	HMG-CoA reductase*	-0.3	-1.0	7.7	12.0	-1.3	-1.5	0.3	0.4	0.5	1.8	-11.2	-1.5
X13722	LDL receptor	-0.1	-0.3	7.3	10.1	-1.6	-1.4	2.0	2.9	2.7	3.1	-4.4	-0.1
U53706	Mevalonate pyrophosphate decarboxylase	-1.1	-2.1	-1.4	-1.3	-1.8	-1.6	-4.0	-1.8	-1.2	-1.2	-4.6	-0.5
A1230294	PPAR-δ	0.3	0.4	1.8	1.9	3.1	2.7	3.5	3.8	7.9	3.7	3.6	2.0
D37920	Squalene epoxidase	-1.1	-1.1	0.2	0.1	-1.5	-2.0	-2.1	-1.7	-1.3	0.0	-3.2	-1.9
AF036761	Stearol-CoA desaturase 2	5.2	2.3	6.5	12.2	-9.9	-9.3	-3.0	-2.0	-1.1	1.5	-3.9	0.6
AA875269	Stearyl-CoA desaturase 2	-1.1	-0.1	-1.4	-1.2	-1.3	-1.3	-1.8	-1.3	-3.1	-1.3	-2.1	-0.3
J05035	Steroid 5 α-reductase	0.0	0.1	-0.4	-0.3	-1.6	-2.7	-11.5	-9.6	-6.8	-6.6	-7.8	-3.6



TABLE 2—Continued

Functional group/GAN	Gene name	1 h		2 h		4 h		8 h		12 h		24 h		
		cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	
5.0 cytokines and chemokines and related receptors	L08497	-2.6	-2.2	1.2	3.0	2.7	2.3	6.2	4.9	6.7	1.6	3.0	-2.1	
	K02813	0.0	1.1	-1.1	0.0	-0.1	-1.2	-1.1	-1.1	-2.3	-1.5	-6.4	0.0	
	AA818097	0.0	-0.1	-1.2	-1.1	-1.5	-1.5	-2.3	-1.9	-2.8	-2.0	-2.6	-1.8	
	M15481	-1.1	-1.1	0.0	0.0	2.1	1.6	2.0	2.7	0.0	1.4	-8.9	-2.0	
	M25584	1.3	-0.2	0.2	0.3	-1.4	-1.6	-2.4	-1.4	-4.8	-2.1	-9.4	-3.9	
	J04807	-1.3	-1.3	-0.2	-1.4	-1.0	1.3	-0.1	-0.3	0.0	0.0	-2.6	-0.5	
	M31076	1.2	-1.1	0.1	0.1	-1.9	-2.9	-3.0	-4.1	-5.5	-1.8	-3.7	-1.4	
	NO independent													
	S77492	0.2	0.1	-0.1	0.0	-2.4	-2.8	-5.3	-5.8	-5.1	-5.2	-2.3	-2.0	
	M11596	1.8	1.6	6.0	6.2	23.0	17.0	45.1	47.1	54.4	58.7	15.3	8.8	
	S54008	1.3	-0.8	-0.7	0.1	-2.9	-2.1	-4.3	-4.5	-1.6	-2.0	1.7	-1.8	
	M91599	1.2	1.1	-1.2	-0.1	-1.7	-1.8	-4.3	-4.0	-3.4	-1.9	-2.0	-1.3	
	AA858520	0.3	0.1	2.4	2.8	3.5	3.2	4.2	3.6	2.7	3.9	0.4	1.5	
	AF058795	-1.1	-0.1	1.2	1.2	0.0	1.1	-3.1	-3.0	-2.5	-2.2	-1.6	-2.3	
	AF076619	0.2	0.0	5.9	9.3	-1.2	1.1	-1.7	-1.9	-1.8	-2.0	-2.0	-1.5	
	Z83757	0.0	0.0	3.2	4.1	-0.4	-0.4	-2.9	-4.4	-2.4	-5.1	-2.1	-2.2	
	U65007	0.2	0.5	1.2	1.4	0.4	1.8	2.7	1.6	2.6	2.9	0.4	1.4	
L29232	-0.4	-0.2	0.0	1.3	2.0	2.4	2.4	3.4	3.4	5.5	1.3	2.0		
M74152	0.2	0.2	-0.1	0.0	-27.0	-2.2	-3.0	-3.6	-4.3	-4.9	-4.7	-3.1		
M64033	-0.4	-0.3	-1.8	-2.0	1.1	-1.1	1.5	1.5	0.0	1.5	-3.8	2.2		
6.0 cytokine processing and signal transduction	NO dependent													
	AJ011969	1.4	2.4	2.0	2.2	0.4	2.8	31.7	13.1	25.2	8.9	6.6	2.8	
	NO independent													
	AA799761	1.8	0.3	8.9	10.5	91.6	73.6	160.7	118.1	111.7	73.7	54.1	21.6	
	D11445	115.8	91.3	166.4	162.3	227.2	247.7	249.1	188.7	190.9	162.7	82.2	51.6	
	AA800602	-1.2	2.2	9.1	10.3	24.7	28.1	85.7	84.8	106.1	40.8	19.3	9.7	
	AF015719	1.6	1.4	1.8	2.5	7.4	6.2	6.3	5.5	6.5	4.1	4.0	4.2	
	D00403	-0.4	0.1	2.2	0.9	8.7	7.0	38.6	26.3	13.2	14.1	-1.6	2.3	
	Z22812	1.4	1.1	1.7	1.8	2.7	1.9	2.2	2.7	2.3	2.1	0.3	1.1	
	XI7053	-0.4	-0.3	0.4	0.6	9.2	6.5	16.7	25.3	4.8	23.5	-0.1	11.0	
	U45965	6.3	5.3	15.0	12.8	39.2	24.4	109.9	73.2	53.2	49.6	9.2	18.9	
	AF053312	1.8	0.5	4.3	4.1	41.7	29.4	87.3	79.4	36.5	35.1	6.1	6.6	
	U17035	29.3	33.0	99.5	85.7	62.4	94.0	69.8	62.2	100.9	82.8	57.2	86.2	
	A1009658	0.1	-0.4	3.4	2.9	-0.5	-1.8	17.1	22.2	22.1	50.2	17.0	13.7	
	E02468	0.4	2.1	4.1	4.7	13.4	7.8	28.9	26.4	11.0	14.2	2.5	4.1	
	L00981	4.7	4.1	10.1	11.3	80.1	39.9	108.8	99.3	48.7	68.6	2.7	12.1	
	NO dependent	CIS	0.1	1.2	7.0	8.8	0.0	0.4	0.2	1.6	2.2	1.6	-3.4	-0.1
COX-2		1.2	1.3	-0.6	-0.3	2.1	1.6	7.0	4.1	4.9	8.6	1.8	2.9	
Delta-1		1.8	2.2	4.5	4.5	5.9	6.1	12.5	7.6	17.3	6.4	3.1	1.9	
Jagged-1*		1.1	1.4	1.7	2.0	6.9	4.2	23.9	12.7	14.2	4.4	9.0	2.1	
Lnkl		1.0	-0.1	0.1	1.2	-1.2	-1.2	2.1	2.0	3.6	2.7	2.6	1.2	
MAP-kinase phosphatase		4.2	3.8	5.5	5.1	5.6	3.7	4.2	4.5	0.0	2.6	-6.4	1.3	

D89863	M-Ras	0.0	0.1	0.0	0.2	3.3	2.9	3.5	2.2	4.1	0.6	5.0	1.2
AB000778	Phospholipase D 1	1.6	0.9	1.6	0.2	2.0	1.9	3.0	2.3	2.1	4.4	1.4	2.5
X63675	Pim-1	-0.1	-0.2	2.1	1.7	1.9	2.2	4.1	2.4	4.2	1.1	2.5	0.0
AF086624	Pim-3 serine threonine kinase	0.2	1.5	2.7	1.5	-2.6	-2.3	1.3	-2.3	5.7	-1.8	6.7	-1.9
A1013987	PKR-RNA-dependent initiation factor-2 kinase	0.5	1.5	2.1	3.2	2.6	3.9	7.0	3.3	4.0	5.8	2.4	1.9
U02553	Protein tyrosine phosphatase	2.8	2.6	13.4	14.4	-0.2	-1.4	2.6	-2.2	2.3	-2.9	-4.5	-1.6
AF075382	SOCS-2	0.3	0.2	2.4	2.8	1.5	1.7	-1.5	-1.4	-2.1	-1.5	-2.5	-0.2
NO independent													
D45920	130kDa-Ins(1,4,5)P3 binding protein	-0.1	0.1	-1.3	-1.1	-1.6	-2.5	2.5	2.2	2.1	-2.0	2.6	2.1
Z18877	2'5' oligoadenylate synthetase	0.1	0.4	-1.1	0.0	2.7	2.3	7.4	12.2	13.4	21.0	4.8	10.3
M74488	Ca <sup>2+</sup> /calmodulin-dependent protein kinase 3	0.6	0.4	1.7	2.0	-2.1	-2.3	-3.6	-3.7	-2.2	-2.5	-2.3	-2.2
D14568	Calcineurin B	-0.1	-0.6	3.5	5.6	0.1	1.5	1.7	0.5	1.3	0.0	1.3	-0.2
L02615	cAMP-dependent protein kinase inhibitor	0.0	0.2	-1.3	1.4	5.7	3.8	2.5	2.4	-0.1	0.5	0.1	-0.3
U03388	COX-1	0.2	-0.1	-1.1	-0.3	1.7	1.4	4.0	4.0	4.6	3.2	2.4	2.4
L02530	Drosophila polarity gene (frizzled) homologue	-0.3	0.0	-1.5	-2.4	-7.0	-7.3	-3.7	-2.4	-3.4	-2.0	0.0	-1.5
M64300	ERK2	0.2	0.0	3.8	5.2	-1.2	-1.2	-1.2	-1.5	1.3	-1.1	0.5	0.0
M64301	ERK3*	1.6	1.6	5.0	5.6	3.2	2.9	4.7	4.1	7.0	3.0	2.4	1.8
S80456	Guanylate cyclase-A	12.7	10.3	21.5	19.0	7.2	8.0	17.9	14.2	8.3	5.6	5.0	5.9
AA891944	INF $\gamma$ induced GTPase	5.2	6.8	33.5	51.0	96.9	88.9	48.5	38.3	26.4	38.0	45.0	57.6
AJ000556	JAK-1	0.1	-0.3	4.2	5.8	0.2	-1.1	-1.2	-1.8	1.5	0.0	1.4	-0.2
U13396	JAK-2	0.7	-1.2	3.2	3.8	11.0	15.9	16.3	16.6	29.5	14.7	9.8	4.8
X52713	Mx3	5.3	4.3	1.2	2.6	0.2	0.0	62.9	64.7	100.4	151.9	48.5	32.2
D28560	Phosphodiesterase 1*	-1.1	-1.1	1.2	1.1	-1.1	0.1	-1.5	-1.7	-1.5	-1.4	-3.7	-2.4
AF027571	Phospholipase C b4	0.1	-0.4	4.8	7.3	-1.3	-2.0	-1.6	-1.9	0.1	-1.5	-2.0	-0.5
U50412	PI3K regulatory subunit p85 $\alpha$	1.7	0.0	3.7	3.5	3.7	2.6	4.0	3.1	8.3	6.5	2.0	1.7
X04139	PKC- $\beta$	0.0	1.1	-1.2	-1.1	0.1	-0.1	2.5	2.7	1.9	1.4	-1.5	-2.0
M18330	PKC- $\delta$	1.2	0.0	2.7	3.0	1.3	1.7	1.6	2.1	3.1	2.0	1.7	1.4
M18331	PKC- $\epsilon$	0.0	0.2	1.9	2.3	0.0	-1.3	-9.5	-6.7	-2.6	-2.0	0.1	0.1
AA800318	Serine protease inhibitor 1	1.0	1.3	4.0	4.2	11.8	12.4	31.6	30.7	30.0	33.1	23.2	42.4
L27128	Stress activated protein kinase $\beta$	0.0	1.1	1.9	1.7	1.8	0.7	7.1	7.0	10.4	4.5	6.4	4.0
AJ012603	TNF $\alpha$ converting enzyme (TACE)*	0.1	-0.2	1.1	1.1	-0.1	-1.2	1.5	1.5	2.8	1.5	-1.4	0.0
7.0 MHC-related													
NO independent													
AF074608	MHC class I antigen (RTL1.EC2)	1.7	1.5	1.6	1.7	3.6	3.9	3.5	2.8	4.4	4.4	4.7	6.0
M36151	MHC class II A-b RT1.B-b- $\beta$	-1.2	1.4	1.7	0.3	-1.9	2.5	3.2	2.8	5.6	13.9	10.5	23.1
X57523	Mtp1*	1.8	1.8	6.2	6.7	10.9	14.8	11.5	9.4	18.3	14.2	13.5	17.4
X63854	Mtp2	-0.1	-0.1	1.6	2.1	4.4	5.1	6.3	9.7	10.8	9.3	10.9	8.3
D45249	Proteasome activator rPA28 subunit $\alpha$	1.1	-1.1	-1.3	-1.2	1.5	1.5	2.4	2.6	2.3	2.8	2.6	3.4
D10757	Proteasome subunit RING 12	-0.1	1.3	3.4	3.4	5.7	7.3	8.1	12.4	13.6	17.7	10.7	21.6
8.0 cell adhesion, cytoskeleton related													
NO dependent													
D00913	ICAM-1*	1.8	1.7	9.0	13.5	29.6	39.4	30.7	29.8	41.2	11.2	9.5	4.1
AA800948	Tubulin $\alpha$ -4	1.6	0.5	-0.1	0.0	-1.1	-1.5	-2.7	-0.2	-6.6	-15.4	-4.8	0.0
AA892333	Tubulin $\alpha$ -6	0.0	0.1	-1.2	-1.3	0.0	-0.2	-1.3	-1.1	-2.9	-1.2	-3.1	-1.3
NO independent													
U75405	$\alpha$ 1 type 1 collagen	1.0	0.3	3.1	3.2	6.9	4.9	4.3	3.8	2.1	2.2	1.6	-0.3
AA891194	ArgBP2	0.1	0.0	-0.6	-1.3	-1.2	-0.1	-2.0	-2.7	-5.7	-6.0	-5.5	-3.0
AF017437	CD-47*	1.2	1.1	2.1	2.3	1.7	1.6	3.3	3.4	3.6	3.4	3.0	3.0
Y16898	Connexin36	2.6	3.1	6.7	8.8	11.9	7.1	7.5	5.7	7.7	5.2	1.4	2.1
X59149	Neural cell adhesion molecule L1	0.3	-0.9	2.7	2.9	1.4	1.4	5.5	6.3	6.3	5.6	4.6	3.3

Continued on following page

TABLE 2—Continued

Functional group/GAN	Gene name	1 h		2 h		4 h		8 h		12 h		24 h	
		cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk
<b>9.0 transcription factors and related genes</b>													
NO dependent													
X64403	C/EBP $\gamma$	1.9	1.0	2.6	3.7	1.7	1.7	2.2	1.4	3.8	1.6	3.6	2.1
S77528	C/EBP $\beta$ *	0.0	-1.4	3.7	3.6	0.3	1.9	4.4	3.1	11.0	6.0	8.1	3.0
X17163	c-jun*	16.6	16.4	8.9	12.9	5.5	9.3	14.2	8.4	13.2	5.9	12.4	3.4
D37951	c-myc intron binding protein 1	0.2	0.0	1.4	1.6	2.2	2.0	2.4	1.6	2.8	1.2	-1.1	-0.1
Y00396	c-myc*	1.2	-1.5	0.1	-0.1	-0.6	-3.1	1.6	-1.9	3.3	-1.8	12.3	0.4
U18982	Fra-2	0.0	-0.3	3.3	3.2	-3.3	-0.8	1.3	3.4	18.9	2.7	4.0	0.3
S69329	Isl-1*	1.0	-1.3	-2.1	-2.3	-2.6	-2.8	-3.5	-2.4	-5.2	-1.6	-5.3	-1.4
U53450	Jun dimerization protein 1	-1.6	0.3	3.6	4.3	2.5	1.0	5.6	5.9	7.2	5.8	4.5	1.8
AA892849	Max dimerization protein 4	-3.8	-2.7	3.9	5.0	-2.8	-1.7	-13.5	-10.0	-4.2	-5.2	-8.3	0.2
AF014503	p8	-0.4	0.6	0.1	1.7	-3.3	-1.2	1.9	0.4	10.4	0.2	24.9	3.3
AF087437	PEBP2 $\beta$	1.1	1.1	0.0	0.1	-1.2	-1.9	-1.4	-1.3	-2.2	-1.3	-2.9	-1.6
X54686	pJunB	25.6	23.9	18.4	21.2	24.1	31.4	44.1	25.3	42.1	16.1	13.2	5.9
AF009329	SHARP-1	-1.3	-1.2	-1.4	-1.6	-1.6	-2.2	-1.5	-2.5	3.0	-1.1	4.7	0.0
AF009330	SHARP-2	-1.1	-1.5	0.3	0.0	0.1	0.2	6.0	1.9	15.5	-3.8	21.1	1.5
AF042499	Smad7	-2.2	0.0	6.5	10.2	2.6	-3.9	9.4	7.6	6.6	3.9	2.6	-0.1
U88630	Spl-like zinc finger protein	1.6	1.7	11.0	15.8	-3.4	-0.5	-0.3	-1.0	1.5	-0.7	-7.7	-3.8
AF026476	USF-1	0.3	0.6	2.5	3.8	0.5	2.3	2.8	4.1	2.8	2.2	4.2	2.9
NO independent													
M65149	C/EBP $\delta$	-0.1	-1.2	1.8	1.3	2.4	2.4	6.6	3.4	8.1	4.3	6.9	5.1
X06769	c-fos*	7.1	5.9	2.3	2.5	0.4	0.6	3.2	3.0	-2.2	0.3	0.0	-1.2
S66024	CREM transcriptional repressor	1.5	0.4	6.9	8.7	-0.2	-2.4	-5.6	-6.7	-3.1	-7.4	-3.2	-4.2
AA892297	Histone deacetylase 2	-1.1	0.1	-1.3	-1.2	0.0	0.0	-1.5	-1.4	-2.9	-1.9	-1.3	-1.4
L13201	HNF-3/forkhead homolog-1	-1.5	-1.5	1.3	1.3	3.6	2.5	4.3	3.5	2.6	2.0	1.8	0.1
L09647	HNF-3 $\beta$	-0.4	-0.4	-2.4	-2.8	-2.9	-3.2	-2.3	-2.0	-1.8	-1.5	0.1	-1.3
X63594	I $\kappa$ B $\alpha$ *	20.6	22.2	38.7	36.7	35.2	39.5	19.1	18.8	27.7	23.0	12.0	21.1
M34253	IRF-1*	106.4	118.5	87.8	74.8	94.1	116.6	85.4	70.6	72.3	48.8	34.7	30.5
AA799861	IRF-7	2.0	2.0	2.4	3.1	5.3	5.7	25.9	36.8	23.3	26.2	14.5	15.6
A1176662	Krox-24	4.8	4.8	2.4	3.2	4.7	8.7	6.7	6.6	3.6	5.7	-1.7	2.0
U56241	Maf-1	-0.8	0.2	-1.4	-1.5	-3.9	-3.0	-4.4	-3.6	-2.2	-2.4	-2.9	-2.2
D82074	NeuroD1/BETA2	1.2	-1.1	-0.1	1.3	-1.7	-1.3	-2.6	-1.9	-2.4	-1.6	-1.5	0.0
D82868	NeuroD2	-0.2	-0.4	-0.7	-0.3	-2.6	-4.8	-2.1	-1.7	-1.1	-0.2	0.2	-1.1
L26267	NF- $\kappa$ B p105	1.3	1.2	6.5	8.0	15.1	12.2	16.4	16.2	16.8	10.9	7.4	7.2
AF004431	Nkx6.1	-1.2	-1.2	-2.1	-2.2	-2.5	-3.3	-3.5	-3.8	-3.3	-3.3	-1.2	-2.2
S74393	Pax-6	1.1	-1.1	-1.5	-1.7	-1.6	-2.8	-1.2	-0.2	-2.1	-1.5	-1.4	-0.1
AF020618	Progression elevated gene 3	3.0	3.2	2.6	2.7	2.4	2.9	3.0	2.4	7.4	1.3	4.2	1.5
AA892553	STAT-1*	0.4	1.6	7.2	8.1	9.4	8.1	15.0	14.0	19.0	19.1	23.6	24.8
X91810	STAT-3	0.5	0.3	6.5	5.4	2.2	2.1	14.1	11.0	7.0	4.2	1.7	2.5
<b>10.0 RNA synthesis and splicing factors</b>													
NO dependent													
AA875129	Elongation factor II	1.1	1.1	0.0	-1.3	-1.3	-1.6	-2.2	-2.1	-3.7	-2.5	-3.2	-1.7
H31550	Polyadenylation element binding protein	1.1	0.0	-1.4	-1.4	1.4	0.0	2.3	2.3	2.7	2.0	4.0	-0.3
NO independent													
U32577	M4 protein homolog	-0.1	0.1	2.6	1.1	-1.4	0.0	-0.3	-0.7	-1.5	0.3	-2.4	-0.1
A1231164	RNA binding protein (transformer-2-like)	1.2	-1.1	4.4	5.8	-0.2	-1.2	1.6	0.3	2.2	0.2	1.8	0.0
AF079873	Splicing factor 1 homolog	-0.3	-0.3	4.5	8.1	-1.3	-1.1	1.7	1.4	1.3	1.2	-1.1	0.1



TABLE 2—Continued

Functional group/GAN	Gene name	1 h		2 h		4 h		8 h		12 h		24 h	
		cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk	cyk	NMA + cyk
AJ010386	ETR-R3b protein	-1.1	0.1	4.1	4.6	0.0	0.1	1.8	2.0	2.1	2.2	0.0	1.9
U84410	ICE-related protease CPP32	1.8	1.0	4.0	5.1	2.2	2.6	1.9	0.8	0.0	1.4	-1.4	1.5
U35890	Polypeptide GalNAc transferase T1	-0.2	0.0	3.8	6.3	-1.4	-0.6	-1.5	-2.0	-1.8	-1.3	-3.2	-2.1
M96630	Sec 61 homolog	0.1	0.0	9.1	15.8	-1.4	-1.2	-0.5	-0.2	-0.1	0.0	-2.1	0.0
AA685903	Similar to GRP94	0.2	0.0	5.9	9.3	-1.2	1.1	-1.7	-1.9	-1.8	-2.0	-2.0	-1.5
X68191	Sodium-calcium exchanger	1.2	-0.3	-1.3	1.9	-0.3	0.1	4.3	2.7	3.6	2.2	1.6	1.7
Z14030	TRAP-complex $\gamma$ subunit	0.2	-1.5	9.2	12.3	0.0	-0.3	-0.1	-1.7	0.0	0.0	1.2	-1.3
14.0 miscellaneous													
NO dependent													
Y07704	Best-5*	-0.5	0.2	2.2	2.3	21.2	21.5	225.0	148.5	182.9	146.7	138.5	30.8
L21711	Galectin-5	-0.4	2.1	1.4	-1.5	2.2	0.9	14.3	19.5	19.8	55.8	12.3	32.6
NO independent													
U95001	Developmentally regulated cardiac factor 5	0.1	-1.1	-1.4	-1.4	-1.7	-2.5	-2.3	-2.3	-2.9	-2.0	-2.1	-1.8

Data are means of two independent microarray experiments and are expressed as fold change versus control cells, which were studied at the same time points. Cytokine-induced differences in gene expression were considered present when the mean fold change of the duplicates was  $\geq 2.5$  and both individual fold-change values were  $\geq 2.0$  in at least one time point studied. Cytokine-modified genes were considered NO dependent if their expression was increased or decreased by  $>50\%$  by NMA in at least one time point and increased or decreased by  $>30\%$  at a second time point. \*Gene represented by more than one probe. cyk, cells exposed to IL-1 $\beta$  + IFN- $\gamma$ ; GAN, Genbank accession number; NMA + cyk, cells exposed to NMA + IL-1 $\beta$  + IFN- $\gamma$ ; -, decreased expression; no sign, increased expression.

**Microarray and clustering analysis.** Total RNA isolated from INS-1E cells (at least 10  $\mu\text{g}/\text{sample}$ ) was used to prepare biotinylated cRNA. The marked cRNA was hybridized to the rat U34-A oligonucleotide array (Affymetrix, Santa Clara, CA) as previously described (8,10). A total number of 48 U34-A arrays were utilized in the present series of experiments. Analysis of differential expression was performed by the GeneChip Suite software (version 4.0.1). Arrays were normalized by global scaling, with the arrays scaled to an average intensity of 150. Samples from duplicate experiments (INS-1E cells from different passage numbers) were hybridized to increase the robustness of the results. Gene expression was considered as modified according to previously described criteria (8,10). Briefly, genes were considered as modified by cytokines when they fulfilled the following criteria for at least one of the six time points studied: 1) the mRNA was present in either control or cytokine-treated cells in both experiments, 2) the mean average fold change (experimental group versus control) was  $\geq 2.5$ , and 3) the fold change in each individual duplicate was  $\geq 2.0$ . We have used our own curated "β-Cell Gene Bank" to assign the filtered genes into their respective functional clusters. The ESTs that had homology to a known sequence were annotated using the Resourcerer 6.0 database (22). A cytokine-modified gene, according to the criteria described above (mean average fold change  $\geq 2.5$  and the fold change in each individual  $\geq 2.0$ ) was considered NO-dependent if its expression in the presence of cytokines was increased/decreased  $>50\%$  by NMA (mean of two experiments) in at least one time point and increased/decreased  $>30\%$  at a second time point (mean of two experiments). These criteria of considering genes as NO dependent is arbitrary and may result in the inclusion of genes that are only partially regulated by the radical. Clustering analysis was performed to classify the genes according to their temporal variation in response to cytokines. For this purpose, the log-transformed expression values of the cytokine-induced mRNAs (genes + ESTs) were mean and variance normalized. The preprocessed data were used as input data in the J-Express clustering software (23), and a distance matrix was created with the Pearson correlation distance measure. K-means clustering method analysis was performed to create 15 clusters after the initial observation of 15 patterns with the hierarchical clustering method (13). The k-means algorithm is explained in detail elsewhere (15,24).

**RT-PCR analysis.** RT-PCR was performed on poly(A)+ RNA as described (10). Primers used for PCR amplification were for notch-1 F-CTCAGCTGATGTCAATGCT, R-GTGTGGGAGACAGAGTGGGT (366 bp); delta-1 F-AAGGCCCGAGTCTGTCTACT, R-TGCTAACTCCGAGATGAACC (256 bp); jagged-1 F-A GCCTGTGAGCCTTCCTTAT, R-AAGCCACTGTTAAGACAGAGC (241 bp). The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were described previously (8). The ethidium bromide-stained agarose gels were photographed under ultraviolet transillumination using a Kodak Digital Science EDAS 290 camera (Eastman Kodak, Brussels, Belgium). The abundance of the PCR products were assessed by Biomax one-dimensional image analysis software (Kodak), and mRNA contents were expressed as optical densities corrected for GAPDH.

**Promoter studies.** Plasmid constructs containing the iNOS and MCP-1 gene promoters were prepared and studied as described previously (25,26). Transfected INS-1E cells were exposed to cytokines with or without NMA for 12–24 h (same concentrations as above). Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega). Test values were corrected for the luciferase activity value of the internal control plasmid, pRL-CMV. The results for cytokine-exposed cells were expressed as a fold induction of the luciferase activity in control condition, taking control (no cytokine added) value as 1. NMA alone did not affect the promoter activity in any of the conditions studied (data not shown).

**Statistical analysis.** Results are given as means  $\pm$  SE. Comparisons versus the respective control groups were performed using the Student's paired *t* test or Wilcoxon's signed-rank test, as indicated.

**RESULTS**

In the initial part of the study, we examined the effects of cytokines on INS-1E cell viability and NO production. Exposure of INS-1E cells to IL-1 $\beta$  + IFN- $\gamma$  for 2 h and 6 h did not affect viability, but after 12 and 24 h, there was an increase in cell death by apoptosis, without significant changes in the percentage of necrotic cells (Fig. 1). The increase in INS-1E cell death was paralleled by progressive medium nitrite accumulation after 12 and 24 h (but not after 2 or 6 h, data not shown), indicating cytokine-induced NO production (Fig. 2). Addition of the iNOS blocker NMA prevented cytokine-induced nitrite produc-

tion (Fig. 2) but did not protect INS-1E cells against apoptosis (Fig. 1). These findings indicate that cytokine-induced apoptosis in INS-1E cells is mostly NO independent. Similar observations were made in human and rodent purified primary  $\beta$ -cells (1,19,20), suggesting that the well-differentiated INS-1 cells are an adequate model for the present experiments.

For the microarray experiments, INS-1E cells were exposed to NMA, IL-1 $\beta$  + IFN- $\gamma$ , NMA + IL-1 $\beta$  + IFN- $\gamma$ , or left untreated (control) for 1, 2, 4, 8, 12, and 24 h. The microarray analysis was performed as previously described (8,10), utilizing the Affymetrix rat U34A oligonucleotide array containing around 8,700 probes (77% known genes and 23% ESTs). Around 4,000 (3,415–4,202) genes and ESTs were scored as present in each of the 48 conditions. To evaluate whether NMA by itself modifies INS-1E cell gene expression, we compared INS-1E cells exposed to NMA for different time points to their respective controls, i.e., INS-1E cells not exposed to NMA but cultured for the same time period. NMA induced changes in 48 probes at the different time points tested (data not shown), i.e., <0.6% of the genes detected as expressed. These genes were excluded from further analysis. These findings indicate that NMA alone has a minor effect on INS-1E cell mRNA expression and suggest that the most relevant effect of NMA on cytokine-induced gene expression is associated to inhibition of NO production (Fig. 2).

After filtering the expression values following the stringent criteria outlined in RESEARCH DESIGN AND METHODS, 936 mRNA probes encoding for 698 known genes and ESTs were detected as cytokine regulated (in the Affymetrix array, there are sometimes 2–4 distinct probes for the same gene or EST). We confirmed by RT-PCR eight selected genes detected as cytokine-modified by the microarray analysis, namely MCP-1, HO, insulin, iNOS, and isl-1 (data not shown), jagged-1, delta-1, and notch-1 (see below). Moreover, we confirmed that, as previously described for primary  $\beta$ -cells (8,10), cytokines induce a nearly 50% decrease in pancreatic duodenal homeobox factor (Pdx)-1 mRNA expression in INS-1E cells after 24 h, an effect prevented by NMA (data not shown).

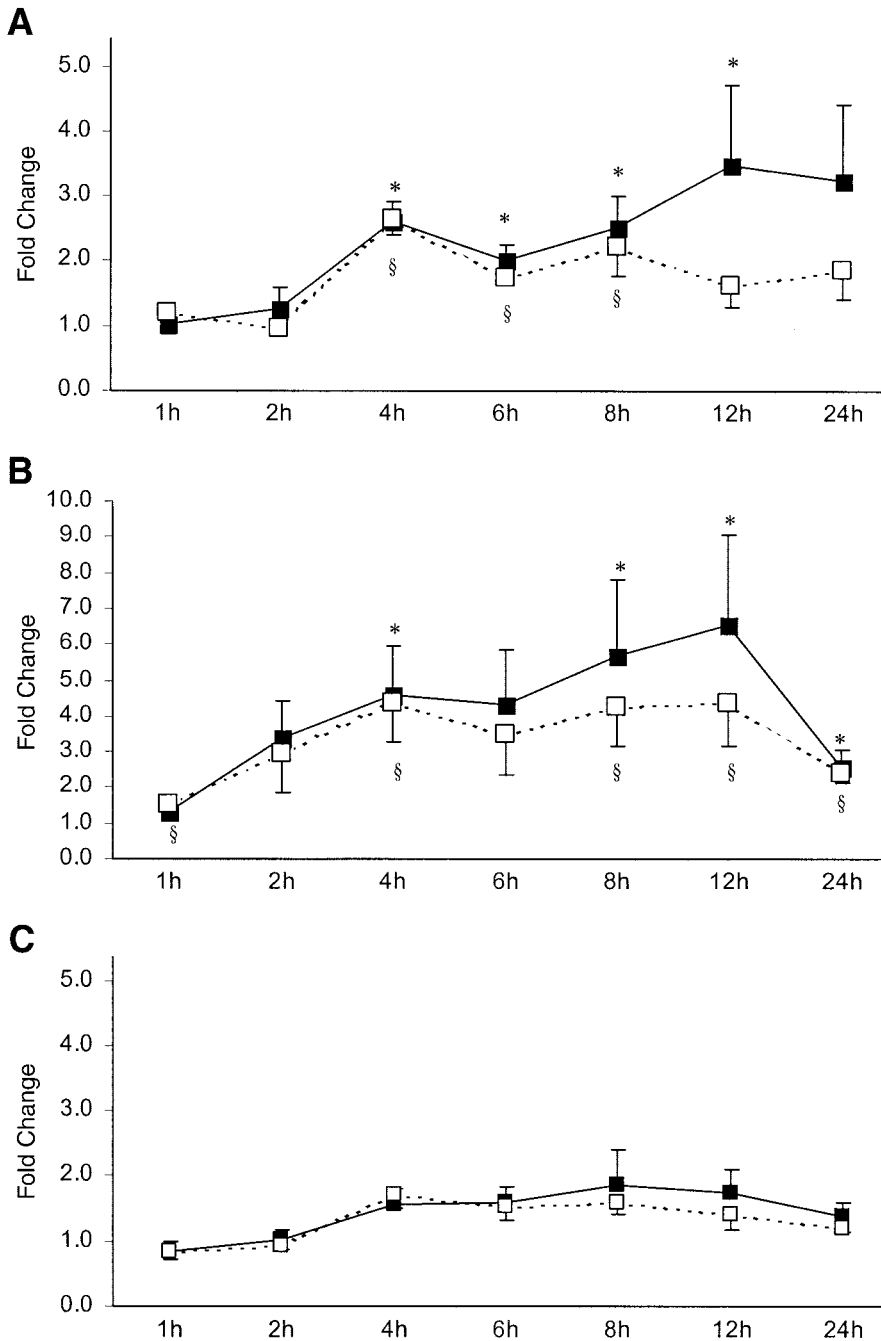
The known genes were further assigned into functional clusters, as shown in Tables 1 and 2 (the total list of genes is provided in an online appendix available at <http://diabetes.diabetesjournals.org>). Of these genes, nearly half were NO dependent. The distribution of NO-dependent/independent genes was ~50% in most functional groups of genes, with the following exceptions. 1) In the metabolism group, 60% of the genes are NO dependent. The most NO-dependent metabolism subgroups are amino acids (67%) and ATP (87%). 2) In the cell cycle group, 71% of the genes are NO dependent. 3) In the cytokine/chemokine and major histocompatibility complex (MHC)-related gene groups, 87 and 100%, respectively, of the genes are NO independent.

NO seems to induce a late (after 12 h) negative feedback for some genes, such as iNOS, argininosuccinate synthetase (AS), MCP-1, and I $\kappa$ B, which is prevented by NMA (Table 2; sections 1.2, 5.0, 9.0). We have previously observed an NO-mediated negative feedback during cytokine-induced iNOS expression in RINm5F cells (27). This negative feedback has been explained in other cell types

by putative effects of the radical on NF- $\kappa$ B activation and promoter regulation (28,29). To test this possibility, we evaluated cytokine-induced iNOS and MCP-1 promoter activity using luciferase reporter constructs (25,26) in the presence or absence of NMA (same experimental conditions as in Fig. 1; exposure time to cytokines and/or NMA of 24 h). Cytokine-induced MCP-1 promoter activity was respectively  $6.4 \pm 2.0$ - and  $4.7 \pm 0.7$ -fold above control level in the presence or absence of NMA (results as means  $\pm$  SE of three experiments), and for iNOS it was  $25.5 \pm 1.7$ - and  $18.7 \pm 2.9$ -fold, respectively, above control level in the presence or absence of NMA (results as means  $\pm$  SE of three experiments). Thus, NMA did not modify cytokine-induced MCP-1 and iNOS promoter activity. NMA also did not interfere with promoter activity in the absence of cytokines (data not shown). These results suggest that the late NO-mediated negative feedback on iNOS and MCP-1 expression in INS-1 cells is not exerted at the level of promoter activity and may be related to mRNA stability and/or another level of regulation still to be determined.

A general analysis of cytokine-modified genes (Table 1) revealed that 19.6% of the filtered genes are metabolism related, making it the most prevalent group (Table 1; section 1.0). Surprisingly, the largest group of metabolism genes were those involved in lipid metabolism (5.4% of the total number of genes; Table 1; section 1.4), with nearly 60% of these genes classified as NO dependent. The transcription factor peroxisome proliferator-activated receptor- $\delta$ , which may enhance lipid uptake (30), was upregulated by IL-1 $\beta$  + IFN- $\gamma$  after 4 h of exposure, and the increased expression was maintained up to 24 h (Table 2; section 1.4). In line with this observation, CD-36 (scavenger receptor class B) and adipophilin, both downstream targets of peroxisome proliferator-activated receptor- $\delta$  (30), were upregulated by IL-1 $\beta$  + IFN- $\gamma$  (Table 2; section 1.4). Cytokines caused a parallel and late decrease in the expression of genes involved in free fatty acid  $\beta$ -oxidation (acetylcoenzyme A dehydrogenase, 1-3-oxacyl-CoA thiolase, peroxisomal enzymes, and carnithine palmitoyl-transferase II) and cholesterol biosynthesis (cytosolic 3-hydroxy 3-methylglutaryl-CoA synthase and reductase and steroid 5 $\alpha$ -reductase) (31) (Table 2; section 1.4). Moreover, cytokines induced genes that may lead to increasing exogenous free fatty acid apport (lipoprotein lipase and LDL receptor) and genes involved in lipid storage (adipophilin) (Table 2; section 1.4).

Cytokines decreased the expression of several genes related to differentiated  $\beta$ -cell functions and preservation of  $\beta$ -cell mass, including Pdx-1, Isl-1, insulin, GLUT2, glucokinase, and diverse receptors for incretins and growth hormones (10) (Table 2). An intriguing finding was the upregulation of jagged-1 and delta-1, with peak of expression after 8–12 h in an NO-dependent manner, followed by a progressive decrease at later time points (Table 2; 6.0). These findings were confirmed by RT-PCR in INS-1E cells (Fig. 3A and B) and primary  $\beta$ -cells (M.I.D. and D.L.E., unpublished data). Jagged-1 and delta-1 are two ligands of the notch receptors (32,33). Notch-1 is expressed in INS-1E cells but remains unchanged following exposure to cytokines as indicated by RT-PCR analysis (Fig. 3C). In good agreement with the RT-PCR data,



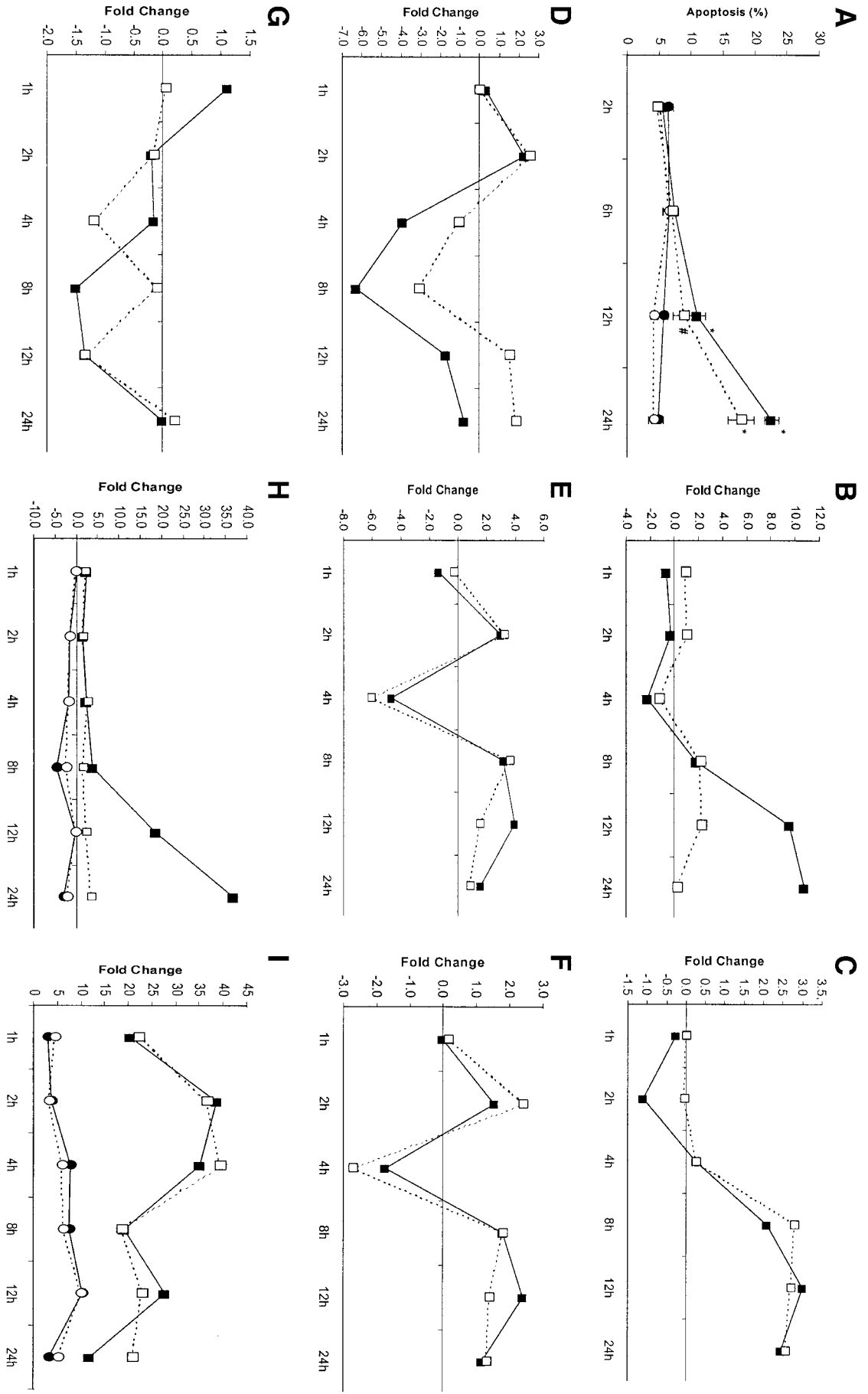
**FIG. 3.** Confirmation by RT-PCR of cytokine-induced modifications in the expression of jagged-1, delta-1, and notch-1 mRNAs. INS-1E cells were cultured for 1–24 h in control condition (no cytokine or NMA added), NMA, IL-1β + IFN-γ (■) or NMA + IL-1β + IFN-γ (□). Jagged-1 (A), delta-1 (B), notch-1 (C), and GAPDH mRNA expression were analyzed by RT-PCR. The values were corrected for the housekeeping gene GAPDH and the mRNA contents expressed as fold change to control condition at the same time point, taking control values as 1. Results are means ± SE of six experiments. \**P* < 0.05 for IL-1β + IFN-γ vs. control; §*P* < 0.05 for NMA + IL-1β + IFN-γ vs. control, Wilcoxon’s signed-rank test. Addition of NMA alone did not modify gene expression at any time point (data not shown).

notch-1 mRNA was detected as “present” but not changed by cytokines in the microarray analysis (data not shown).

It has been previously suggested (8,34) that β-cell exposure to elevated NO concentrations, either via NO donors or cytokines, leads to endoplasmic reticulum stress and consequent β-cell apoptosis. The endoplasmic reticulum stress response is based on the coordinated expression of several different genes. The present time-course microarray experiments provided interesting insights into the parallel up- and downregulation of this gene network (Table 2; section 13.0) (Fig. 4H) and their temporal correlation with β-cell apoptosis (Fig. 4A). Thus, we observed an important (>15-fold) increase in growth arrest and DNA damage (GADD)153/CHOP expression after 12 h, which was prevented by the iNOS blocker NMA (Fig. 4H). Notably, there was also an early (1–8 h) and more modest

(two- to threefold) increase of GADD153/CHOP expression, which seems to be independent of NO formation (Table 2). The late increase in GADD153/CHOP may be secondary to sarco(endo)plasmic reticulum Ca<sup>+2</sup> ATPase type 2 b (SERCA2b) inhibition, which is first detected after 8 h exposure to cytokines, and is also NO dependent (Fig. 4H; Table 2). Cytokine-induced β-cell apoptosis increased in parallel with GADD153/CHOP expression (Fig. 4A) but, contrary to the observations with SERCA2b and GADD153/CHOP, apoptosis was not prevented by the iNOS blocker NMA.

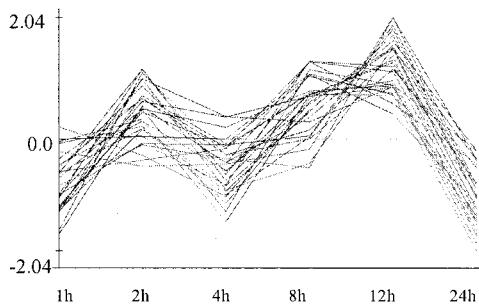
Complex formation between pro- and antiapoptotic proteins from the Bcl-2 family may prevent apoptosis (35,36). Against this background, we examined the expression of known pro- and antiapoptotic genes from the Bcl-2 family (Table 2; section 13.0) (Fig. 4). Exposure of INS-1E



**FIG. 4.** Cytokine-induced gene expression of selected apoptosis- and defense-related genes. **A:** Percentage of apoptotic cells. ○, control; ●, NMA; ■, IL-1β + IFN-γ; □, NMA + IL-1β + IFN-γ. **B–I:** Data as fold variations versus controls; IL-1β + IFN-γ, filled symbols; NMA+IL-1β + IFN-γ, empty symbols. **B:** Bid. **C:** BAK. **D:** BAD. **E:** Bel-xL. **F:** Bel-xS. **G:** Bel-2. **H:** GADD153/CHOP (squares) and SERCA2b (circles). **I:** MnSOD (circles) and IκB (squares). Note the different scales in the diverse figures. Results are means ± SE for four independent experiments in **A**, and the means of two independent microarray experiments in **B–I** (numerical values are indicated in Table 2).

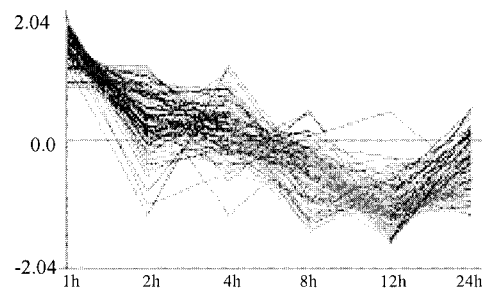
### Cluster 1

NO diag %	T1D %	Met	Prot Synt	Ins Ch	Hum / GF	Cyt / Chem	Sig Tr	MHC	Cell Adh	Tr Fact	RNA Syn	Cell Cyc	Dnf / Resp	Apopt / ER st	Misc
26	23	3	0	3	3	1	8	0	1	4	0	0	0	0	0
3.3	13.0	0.0	13.0	13.0	4.3	34.8	0.0	4.3	17.4	0.0	0.0	0.0	0.0	0.0	0.0



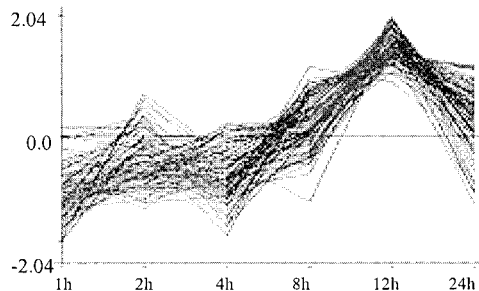
### Cluster 2

NO diag %	T1D %	Met	Prot Synt	Ins Ch	Hum / GF	Cyt / Chem	Sig Tr	MHC	Cell Adh	Tr Fact	RNA Syn	Cell Cyc	Dnf / Resp	Apopt / ER st	Misc
39	52	18	2	4	8	0	5	0	1	7	0	1	1	0	5
7.4	34.6	3.8	7.7	15.4	0.0	9.6	0.0	1.9	13.5	0.0	1.9	1.9	0.0	9.6	



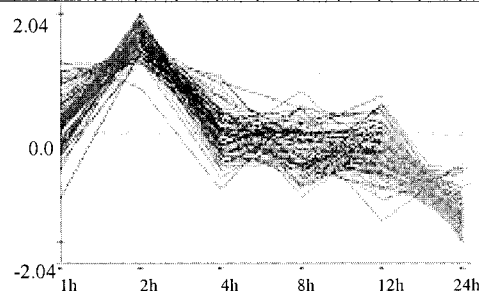
### Cluster 3

NO diag %	T1D %	Met	Prot Synt	Ins Ch	Hum / GF	Cyt / Chem	Sig Tr	MHC	Cell Adh	Tr Fact	RNA Syn	Cell Cyc	Dnf / Resp	Apopt / ER st	Misc
50	72	8	2	5	4	1	8	8	7	12	0	2	4	3	8
10.3	11.1	2.8	6.9	5.6	1.4	11.1	11.1	9.7	16.7	0.0	2.8	5.6	4.2	11.1	



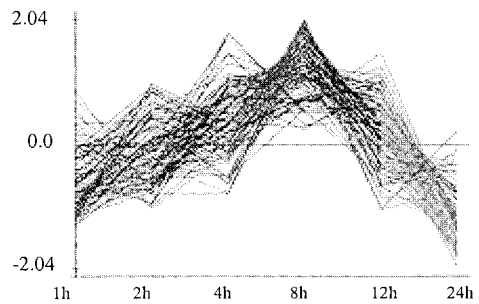
### Cluster 4

NO diag %	T1D %	Met	Prot Synt	Ins Ch	Hum / GF	Cyt / Chem	Sig Tr	MHC	Cell Adh	Tr Fact	RNA Syn	Cell Cyc	Dnf / Resp	Apopt / ER st	Misc
59	32	7	1	2	5	0	5	0	2	6	0	1	3	0	0
4.6	21.9	3.1	6.3	15.6	0.0	15.6	0.0	6.3	18.8	0.0	3.1	9.4	0.0	0.0	



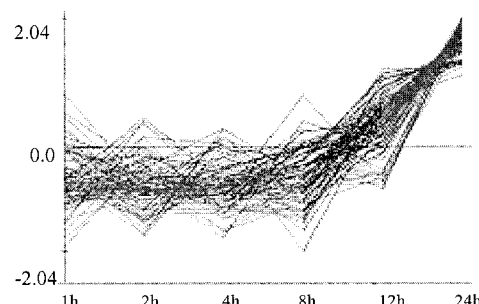
### Cluster 5

NO diag %	T1D %	Met	Prot Synt	Ins Ch	Hum / GF	Cyt / Chem	Sig Tr	MHC	Cell Adh	Tr Fact	RNA Syn	Cell Cyc	Dnf / Resp	Apopt / ER st	Misc
27	44	3	4	1	3	8	10	2	2	5	0	3	0	1	2
6.3	6.8	9.1	2.3	6.8	18.2	25.0	4.5	4.5	11.3	0.0	6.8	0.0	2.3	4.5	



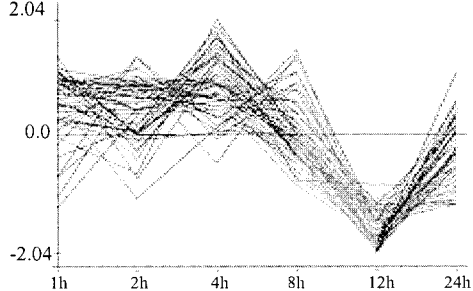
### Cluster 6

NO diag %	T1D %	Met	Prot Synt	Ins Ch	Hum / GF	Cyt / Chem	Sig Tr	MHC	Cell Adh	Tr Fact	RNA Syn	Cell Cyc	Dnf / Resp	Apopt / ER st	Misc
75	59	12	4	2	3	0	5	9	0	10	0	3	4	4	3
8.5	20.3	6.8	3.4	5.1	0.0	8.5	15.3	0.0	16.9	0.0	5.1	6.8	6.8	5.1	



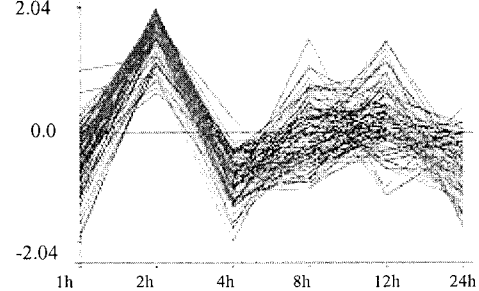
### Cluster 7

NO diag %	T1D %	Met	Prot Synt	Ins Ch	Hum / GF	Cyt / Chem	Sig Tr	MHC	Cell Adh	Tr Fact	RNA Syn	Cell Cyc	Dnf / Resp	Apopt / ER st	Misc
41	44	11	3	2	5	0	2	0	2	8	0	2	3	0	6
6.3	25.0	6.8	4.5	11.4	0.0	4.5	0.0	4.5	18.2	0.0	4.5	6.8	0.0	13.6	



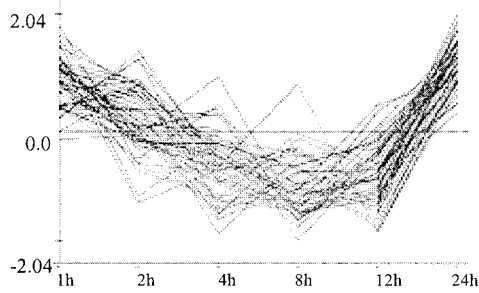
### Cluster 8

NO diag %	T1D %	Met	Prot Synt	Ins Ch	Hum / GF	Cyt / Chem	Sig Tr	MHC	Cell Adh	Tr Fact	RNA Syn	Cell Cyc	Dnf / Resp	Apopt / ER st	Misc
45	85	20	9	4	7	0	9	0	4	3	2	6	3	7	11
12.2	23.5	10.6	4.7	8.2	0.0	10.6	0.0	4.7	3.5	2.4	7.1	3.5	8.2	12.9	



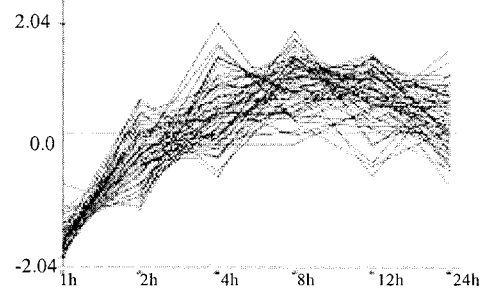
Cluster 9

	NO dep %	T.N./%	Met	Prot.Synth	Ion Ch	Horm / GF	Cyt / Chem	Sig Tr	MHC	Cil Adh	Tr Fact	RNA Syn	Cil Cyc	Def / Resp	Apop / ER st	Misc
45	20	7	0	1	3	0	4	0	0	4	0	0	1	0	0	
	2.9	35.0	0.0	5.0	15.0	0.0	20.0	0.0	0.0	20.0	0.0	0.0	5.0	0.0	0.0	



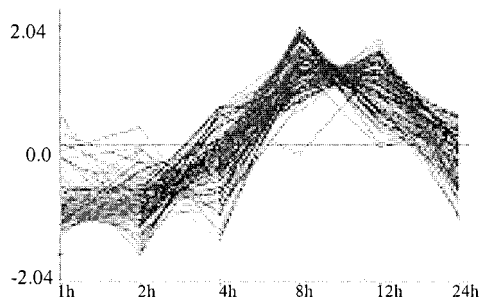
Cluster 10

	NO dep %	T.N./%	Met	Prot.Synth	Ion Ch	Horm / GF	Cyt / Chem	Sig Tr	MHC	Cil Adh	Tr Fact	RNA Syn	Cil Cyc	Def / Resp	Apop / ER st	Misc
9	33	4	0	0	4	5	5	2	4	5	0	1	1	1	1	
	4.7	12.1	0.0	0.0	12.1	15.2	15.2	6.1	12.1	15.2	0.0	3.0	3.0	3.0	3.0	



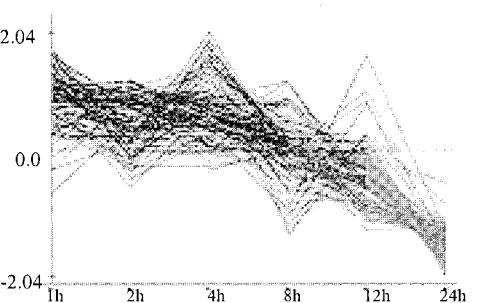
Cluster 11

	NO dep %	T.N./%	Met	Prot.Synth	Ion Ch	Horm / GF	Cyt / Chem	Sig Tr	MHC	Cil Adh	Tr Fact	RNA Syn	Cil Cyc	Def / Resp	Apop / ER st	Misc
32	78	6	6	2	8	3	21	9	3	8	0	3	4	1	4	
	11.2	7.7	7.7	2.6	10.3	3.8	26.9	11.5	3.8	10.3	0.0	3.8	5.1	1.3	5.1	



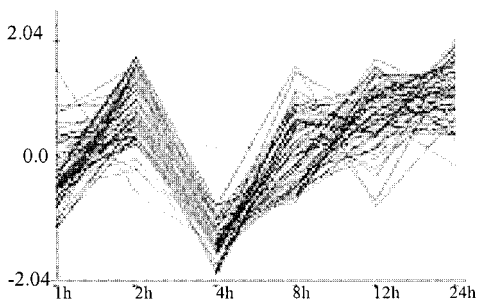
Cluster 12

	NO dep %	T.N./%	Met	Prot.Synth	Ion Ch	Horm / GF	Cyt / Chem	Sig Tr	MHC	Cil Adh	Tr Fact	RNA Syn	Cil Cyc	Def / Resp	Apop / ER st	Misc
60	65	12	4	1	5	0	8	0	5	9	2	5	6	0	8	
	9.3	18.5	6.2	1.5	7.7	0.0	12.3	0.0	7.7	13.8	3.1	7.7	9.2	0.0	12.3	



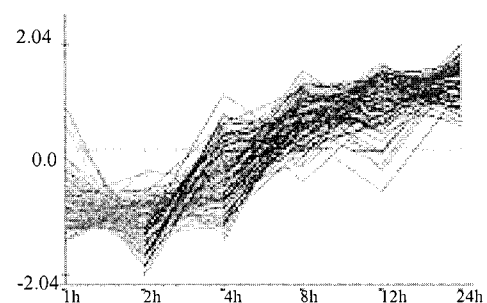
Cluster 13

	NO dep %	T.N./%	Met	Prot.Synth	Ion Ch	Horm / GF	Cyt / Chem	Sig Tr	MHC	Cil Adh	Tr Fact	RNA Syn	Cil Cyc	Def / Resp	Apop / ER st	Misc
51	33	4	4	2	4	0	6	1	3	2	1	1	3	1	1	
	4.7	12.1	12.1	6.1	12.1	0.0	18.2	3.0	9.1	6.1	3.0	3.0	9.1	3.0	3.0	



Cluster 14

	NO dep %	T.N./%	Met	Prot.Synth	Ion Ch	Horm / GF	Cyt / Chem	Sig Tr	MHC	Cil Adh	Tr Fact	RNA Syn	Cil Cyc	Def / Resp	Apop / ER st	Misc
47	32	3	1	1	1	0	2	3	2	4	3	0	2	3	7	
	4.6	9.4	3.1	3.1	3.1	0.0	6.3	9.4	6.3	12.5	9.4	0.0	6.3	9.4	21.9	



Cluster 15

	NO dep %	T.N./%	Met	Prot.Synth	Ion Ch	Horm / GF	Cyt / Chem	Sig Tr	MHC	Cil Adh	Tr Fact	RNA Syn	Cil Cyc	Def / Resp	Apop / ER st	Misc
46	26	4	3	0	1	0	7	0	1	4	0	0	2	2	2	
	3.7	15.4	11.5	0.0	3.8	0.0	26.9	0.0	3.8	15.4	0.0	0.0	7.7	7.7	7.7	

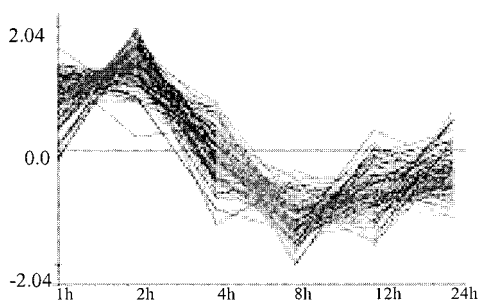


FIG. 5. K-means clustering analysis of cytokine-induced gene expression. Mean- and variance-normalized data (y-axis) were used to create 15 clusters. The functional classification in each cluster is indicated at the top of the figure. NO dep %, percentage of NO-dependent probes in the cluster; T.N./%, total number of probes in the cluster/percentage of the total number of probes modified by cytokines; Met, metabolism; Prot Synth; protein synthesis; Ion Ch, ionic channels; Horm/GF, hormones and growth factors; Cyt/Chem, cytokines and chemokines; Sig Tr, signal transduction; MHC, MHC related; Cil Adh, cell adhesion; Tr Fact, transcription factors; RNA Syn, RNA synthesis, Cil Cyc, cell cycle; Def/Resp, defense/repair; Apop/ER st, apoptosis and ER stress; Misc, miscellaneous.

cells to cytokines modified expression of several proapoptotic genes, including Bid, Bad, Bak, and Bcl-xs. The apoptotic inducer Bax was present in the array but was not changed by cytokines (data not shown). The two genes most consistently induced by cytokines were Bid and Bak. Both mRNAs increased after 8 h and maintained increased expression for up to 24 h. Although Bid induction was prevented by NMA (Fig. 4B), the iNOS blocker did not affect Bak expression (Fig. 4C). Interestingly, the proapoptotic protein Bcl-xs (Fig. 4F), a splice variant of Bcl-xL (Fig. 4E), showed a similar profile as Bcl-xL, including decreased expression at 4 h and subsequent NO-independent increase in expression at later time points.

Two genes that may participate in  $\beta$ -cell defense against cytokines are manganese superoxide dismutase (MnSOD) and I $\kappa$ B, both with the property of decreasing NF- $\kappa$ B activation (37,38). MnSOD and I $\kappa$ B mRNAs were already induced by cytokines after 1 h (Fig. 4I) in an NO-independent manner and maintained an increased expression during the 24-h follow-up. Despite the upregulation of I $\kappa$ B and MnSOD, and of other putative defense/repair genes such as HO, heat shock protein (hsp)70, Gas-6, and glutathione-S-transferase (Table 2; section 12.0), prolonged exposure of  $\beta$ -cells to IL-1 $\beta$  and IFN- $\gamma$  culminates in apoptosis, as shown in Fig. 1.

To further examine the general pattern of cytokine-modified genes, all mRNAs described in online supplement S1 (available at <http://diabetes.diabetesjournals.org>) were reclassified based on their temporal pattern of gene expression, using the k-means clustering method (Fig. 5). (The complete list of genes in the different clusters is outlined in online supplement S2, available at <http://diabetes.diabetesjournals.org>.) To decide on the number of clusters to be used, we initially performed hierarchical clustering and observed 15 major profiles of gene expression. To further validate this selection, we performed k-means clustering using 14 or 16 clusters and obtained similarly shaped profiles (data not shown). Genes related to signal transduction and transcription factors were present in all clusters at similar proportions (mostly around 10–20% of the total number of genes). Examination of the clusters reinforces the notion that NO, which starts to be synthesized in large amounts after 6–8 h, is crucial for late-expressed genes. Thus, the two most “NO-related clusters” were clusters 6 and 12 with 75 and 60%, respectively, of the genes listed as NO dependent (Fig. 5) (online supplement S2). In both cases, the major variation in gene expression, either stimulation (cluster 6) or inhibition (cluster 12), was observed between 8–24 h. Cluster 6 contains a large proportion of MHC-related genes, whose expression peaks at around 8–12 h. These genes are mostly IFN- $\gamma$ -induced and NO independent (10). The other cluster containing a high proportion of MHC-related genes is cluster 11, with a transitory peak of gene expression at 8–12 h and with only 32% of the genes as NO dependent. If we remove the HLA-related genes from the calculation in cluster 6, the number of NO-dependent genes climbs to 86%. Clusters 6 and 12 also contain a high proportion of “defense/repair” (around 9% of the total) genes but not of apoptosis-related genes. The highest number of apoptosis/endoplasmic reticulum stress-related genes (8.2%) is located in cluster 8, a cluster characterized by early (2 h) and transitory

increase in gene expression. This cluster also contains the highest number of genes related to cell cycle (six genes). As an example of apoptosis-related genes, both caspase 3 and voltage-dependent anion channel (VDAC) are present in cluster 8 (online supplement S2; section 13.0). They have an NO independent peak of expression after 2 h (3.7- to 8.1-fold increase) and return to basal levels after 4–8 h. Most cytokines, chemokines, and adhesion molecules are classified in the same clusters (clusters 5 and 10).

## DISCUSSION

We have currently detected nearly 700 cytokine-affected mRNAs in INS-1E cells. To a major extent, these observations broaden the pool of known cytokine-modified genes in insulin-producing cells, in addition to providing for the first time a comprehensive view of cytokine-induced and NO-dependent genes in these cells. Of these genes, nearly 50% were NO dependent, emphasizing the role for this radical in the late (i.e., after 6–8 h, the time point when increased nitrite accumulation is first detected) (39) (data not shown) effects of cytokines on insulin-producing cells. Synthesis of NO in primary  $\beta$ -cells is regulated by an “NO production module” (8,10,40), which includes, among others, iNOS (upregulated after 4 h, with additional increase after 8–12 h, followed by an NO-dependent decrease after 24 h) and AS, an enzyme that recycles citrulline into arginine, allowing continuous NO production (40,41). AS was also upregulated by cytokines with a similar temporal pattern as iNOS (Table 2; section 1.2). We identified another potential component of the “iNOS production module” in insulin-producing cells, namely the enzyme guanosine triphosphate (GTP) cyclohydrolase I (GTPCH). GTPCH is the initial and rate-limiting enzyme in the production of tetrahydrobiopterin, which serves as a cofactor for iNOS in pancreatic islets (42) and other tissues (43,44). Notably, increased expression of GTPCH has been observed in the target tissues of an animal model of autoimmune arthritis (45), and it will be of interest to evaluate whether this is also the case for islet cells from nonobese diabetic mice and BB rats.

Microarray analysis is a precise and reproducible technique to measure the dynamics of a genetic network at the mRNA level (46,47). The rate of confirmation by semiquantitative RT-PCR for 43 cytokine-modified genes detected in our previous (8,10) (J. Rasschaert, D. Liu, A.K.C., B.K., M.K., T.Ø., and D.L.E., unpublished observations) and present microarray studies is >90%. This rate of confirmation increases to >95% with the use of real-time PCR (B.K., A.K.C., M.I.D., M.K., N.M., T.Ø., and D.L.E., unpublished observations). An important issue is whether the observed modifications in mRNA expression correspond to changes in protein expression. This is often the case, as suggested by the following observations. 1) Studies on the expression of mRNA and protein/enzyme activity for specific cytokine-modified genes in  $\beta$ -cells, such as insulin, MnSOD, AS, ornithine decarboxylase, Pdx-1, hsp70, iNOS, A20, MCP-1, IL-15, MIP-3 $\alpha$ , and IP-10 showed a good correlation between these parameters (8–10,40,48–55). 2) Total protein biosynthesis is not inhibited in rat  $\beta$ -cells or mouse islets exposed to cytokines for 24 h (50,56). If mRNA and protein expression often correlates well, as mentioned above, how can we explain that proteomic studies on

IL-1–exposed neonatal rat pancreatic islets (57,58) failed to identify most of the genes described as modified in our present and previous microarray analysis (8,10)? Notably, these proteomic analyses (57,58) also failed to detect many cytokine-induced proteins previously identified as modified by Western blot and enzyme-linked immunosorbent assay, including abundantly expressed proteins, such as insulin (50), iNOS (51), MnSOD (48), GADD153/CHOP (A.K.C., F. Ortis, and D.L.E., unpublished observations), and the chemokines MCP-1 (52), IL-15 (9), MIP-3 $\alpha$  (9), and IP-10 (9). This suggests that the proteomic approach utilized in these previous studies (57,58), although clearly of interest, is not yet sensitive enough to allow adequate comparison with the sensitive and reproducible microarray approach.

We have previously observed by microarray analysis and RT-PCR that cytokines decrease the expression of several genes related to differentiated  $\beta$ -cell functions and preservation of  $\beta$ -cell mass, including Pdx-1, Isl-1, insulin, GLUT2, glucokinase, and diverse receptors for incretins and growth hormones (8,10). Our present data confirm and extend these findings, providing a broader picture of the genes potentially involved in  $\beta$ -cell dedifferentiation during an immune-mediated assault. An intriguing and novel finding was the upregulation of jagged-1 and delta-1. Jagged-1 and delta-1 are two ligands of the notch receptors (32,33), and we observed that notch-1 is expressed in INS-1E cells but remains mostly unchanged following exposure to cytokines. Notch signaling plays an important role in the control of embryonic endodermal endocrine development (59). This pathway activates the expression of Hes genes, encoding transcription repressors, which in turn inhibit expression of genes promoting endocrine differentiation, such as neurogenins (33). Differentiation is repressed in the endocrine precursor cells expressing the activated notch receptors, whereas the signaling cells are free to express neurogenins and differentiate into endocrine cells (33). In line with this model, expression of NeuroD1/BETA2 and NeuroD2, transcription factors that induce and maintain the differentiated state of insulin-producing  $\beta$ -cells, was downregulated in response to cytokines (Table 2; section 9.0). The targets of NeuroD1/BETA2, namely glucokinase (60), insulin (61), secretin (62), and glucagon (63), were also decreased in the present experiments (Table 2; sections 1.1 and 4.0). The cytokine-induced reexpression of jagged-1 and delta-1, and of other genes potentially involved in the notch signaling pathway, raises the question of the putative effects of this pathway in differentiated  $\beta$ -cells exposed to an autoimmune attack. Two possibilities are a contributory role for the loss of the differentiated  $\beta$ -cell phenotype and prevention, in a paracrine fashion, of the differentiation of newly generated  $\beta$ -cells formed as a compensatory response to progressive  $\beta$ -cell destruction (64). Of note, in another model of autoimmune disease, multiple sclerosis in mice, cytokine-induced reexpression of the notch pathway prevents maturation of oligodendrocytes and hence efficient remyelination of axons in the multiple sclerosis lesions (65).

The relative concentration of pro- and antiapoptotic proteins from the Bcl-2 family may decide cellular outcome following some proapoptotic stimuli (35,36). The two Bcl-2-related genes most consistently induced by

cytokines in the present experiments were Bid and Bak. Bid, a proapoptotic protein, is cleaved by caspase 8 and increases mitochondrial permeability by releasing Bax-like factors from Bcl-2 and stimulating the oligomerization and membrane insertion of Bax and/or Bak (36). Bax forms tetrameric channels in the outer membrane of the mitochondria, allowing the release of apoptogenic factors such as cytochrome c from the mitochondrial intermembrane space (35,36). Many forms of cell death require either Bax or Bak (66). The proapoptotic activity of Bak is mostly neutralized by binding to Bcl-xL, whereas Bax is inhibited to a major extent by Bcl-2 (35,36). Taking this into account, we examined the ratios of Bak/Bcl-xL and Bax/Bcl-2 expression in cytokine-treated cells, utilizing the absolute values of expression obtained in the arrays (data not shown). The ratios of Bak/Bcl-xL showed a sharp increase after 4 h (13.2 and 15.1, respectively, as compared with 2.7 and 4.1, respectively, in control;  $n = 2$  similar experiments), returning to basal levels at later time points. On the other hand, cytokines did not change the ratios Bax/Bcl-2 at any of the different time points studied (data not shown) and also failed to decrease Bcl-2 expression. Two other genes found upregulated in the initial hours of cytokine exposure and potentially related to apoptosis are caspase 3 and VDAC. Caspase activation is partially regulated at the transcriptional level, and upregulation of caspases contributes to cell death in chronic neurological diseases (67). VDAC is located in the outer mitochondrial membrane, and participates in the formation of the mitochondrial permeability transition pore, allowing the release of proapoptotic molecules and the sequential activation of caspases 9 and 3 (35,36). The observed early (2–4 h) upregulation of caspase 3, VDAC, and the ratio of the expression of Bak/Bcl-xL may predispose the  $\beta$ -cells to enter the apoptosis program, pending additional stimuli.

To further examine the general pattern of cytokine-modified genes, all mRNAs described in online supplement S1 were reclassified based on their temporal pattern of gene expression, using the k-means clustering method. K-means is an efficient clustering method to process large datasets (24), and it has been successfully used to determine time-course gene expression data in synchronized yeast (15) and in mouse adipocytes (68). Genes related to signal transduction and transcription factors were present in all clusters at similar proportions (mostly around 10–20% of the total number of genes). This homogeneous dispersion is puzzling; we expected transcription factors to be mostly present in “early induced” clusters (1–2 h). If we bear in mind, however, that rodent  $\beta$ -cells continuously exposed to cytokines go through different functional phases, including stimulation in the first 1–3 h, functional inhibition after 6–8 h (69) and progressive cell damage after 8–12 h (1), it becomes evident that  $\beta$ -cells will need to continuously activate diverse transcription factors and signal transduction mechanisms to adapt to the continuous changes in internal homeostasis induced by cytokines. We also observed that two of the clusters, namely clusters 5 and 10, contain many genes previously described as NF- $\kappa$ B dependent (10). For instance, we have shown by detailed promoter studies that NF- $\kappa$ B regulates transcription of iNOS (present in cluster 5) (25), MnSOD (70) (present in cluster 10), and MCP-1 (26) (present in cluster

10). Moreover, the chemokine cytokine-induced neutrophil chemoattractant-1 and the cytokine IL-15, whose expression is at least partially prevented by an NF- $\kappa$ B blocker in  $\beta$ -cells (10), are also present in these clusters, as is also the case for IL-1 $\alpha$ , tumor necrosis factor- $\beta$ , and cyclooxygenase-2, three genes shown to be NF- $\kappa$ B dependent in other tissues (71). Temporal coexpression studies may provide useful information on the nature of the transcription factors regulating groups of genes relevant for  $\beta$ -cell fate, and we intend to perform "in silico" analysis (72) to search for binding sites for key transcription factors present in genes from the different clusters.

We have presently conducted the first systematic time course microarray and cluster analysis of cytokine-exposed insulin-producing cells in the presence or absence of the iNOS blocker NMA. The results obtained increased by more than twofold the number of known cytokine-modified genes, and also provide the first comprehensive analysis of cytokine-induced and NO-dependent mRNAs in insulin producing cells. This collection of genes and gene clusters is an exciting resource for researchers interested in understanding the functional inhibitory and proapoptotic effects of cytokines in  $\beta$ -cells. Moreover, these data provide novel and often surprising insights into the molecular patterns triggered in  $\beta$ -cells faced with a protracted immune assault.

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