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# Involvement of C-Abl Tyrosine Kinase in Lipopolysaccharide-Induced Macrophage Activation<sup>1</sup>

Quan Le, Rene Daniel, Siu-Wah Chung, Anthony D. Kang, Toby K. Eisenstein, Barnet M. Sultzer, Henry Simpkins, and Peter M. C. Wong<sup>2</sup>

LPS endotoxin-induced macrophage activation is recognized to be important in both nonspecific immunity and endotoxin-induced sepsis when excessive macrophage stimulation occurs. In this study, we showed that reduction of c-Abl in macrophages prevented LPS-induced growth arrest, nitric oxide production and TNF- $\alpha$  secretion by ANA-1 macrophages. These cells continued to grow but later underwent apoptosis. Reduction of c-Abl in these cells led to reduced c-Abl kinase activity associated with Ran, which recently has been shown to be an LPS-responsive gene product. Our data suggest that c-Abl tyrosine kinase is one of the intermediates downstream of the initial signal transduction event related to activation of macrophages by LPS. *The Journal of Immunology*, 1998, 160: 3330–3336.

Stimulation of macrophages with LPS endotoxin, a component of the outer membrane of Gram-negative bacteria, results in the production of various cytokines, including TNF- $\alpha$ , IL-1 (1), IL-6, prostanooids, leukotrienes (2), and nitric oxide (NO)<sup>3</sup> (3). Generally, the overproduction of these cytokines is thought to play a significant role in endotoxin-induced septic shock. For this reason, among others, signal transduction in macrophages and B lymphocytes activated by LPS has been under intense investigation in recent years.

Multiple LPS-mediated signal transduction pathways may exist. Interaction of LPS with cells results in activation of pertussis toxin-sensitive guanine nucleotide-binding proteins (4–7). Activation of the Ca<sup>2+</sup> and phospholipid-dependent protein kinase C, which is a serine/threonine kinase, has also been involved in LPS signal transduction (8) and in the induction of tumoricidal properties in macrophages (9–11). Protein tyrosine kinases (PTK) play a central role in regulating cell growth and differentiation and have also been implicated in LPS-mediated signal transduction. The use of specific PTK inhibitors, namely genistein (12, 13), tryphostin (14), and herbimycin A (15), has been shown to block tumoricidal activation of macrophages by LPS (9, 16–19). Which PTK is specifically involved in LPS signal transduction is unknown.

We have recently isolated an LPS-responsive gene that encodes for Ran/TC4 GTPase (20), and is mutated in the genome of LPS hyporesponder C3H/HeJ mice (Wong et al., manuscript in preparation). We have also shown that type I c-abl is involved in LPS-

mediated differentiation of 70Z/3 lymphoid cells (21); LPS stimulation leads to increased levels of type I c-Abl/Ran complex, which enhances the tyrosine kinase activity of the bound type I c-Abl.<sup>4</sup> In this manuscript, we show that c-Abl tyrosine kinase is also involved in LPS-mediated activation of macrophages; reduction of c-Abl leads to inhibition of macrophage activation induced by LPS and induction of apoptosis in macrophages treated by LPS.

## Materials and Methods

### Cell culture and viral infection

The ANA-1 immortalized murine macrophage cell line was established previously by infecting normal bone marrow cells of C57BL/6 mice with the murine recombinant J2 retrovirus containing the v-myc and v-raf oncogenes (22), kindly provided by Dr. Luigi Varesio (National Cancer Institute, Frederick, MD). The cells were cultured in DMEM (Mediatech, Washington, DC) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM L-glutamine, and 10  $\mu$ g/ml gentamicin (D10F). A total of 5  $\times$  10<sup>5</sup> cells/ml were prepared the day before each experiment, and the cells were incubated at 37°C in a humidified incubator with an atmospheric content of 5% CO<sub>2</sub>.

The  $\alpha$ D retrovirus vector was constructed previously (23) and contains a 0.7-kb c-abl antisense sequence and a neomycin resistance (*neo*) gene. The N2 retrovirus carries only a *neo* gene (24). The GP/E virus-producing cells were transfected with plasmid DNA by calcium-phosphate coprecipitation (25), cultured with D10F in 100-mm tissue culture dishes to near confluence, and replaced with 5 ml of fresh D10F. Twelve hours later, the medium was harvested and used as viral supernatant. Aliquots of the viral supernatant were stored at -70°C until use. To determine viral titer, 100,000 NIH 3T3 cells were seeded onto each 60-mm dish. After 12 h, 0.5 ml of viral supernatant with appropriate viral titer and 10  $\mu$ g/ml of polybrene was added to each dish, which was incubated for 2 h, with frequent rocking of dishes. At the end of the incubation/infection, the spent viral supernatants were replaced with fresh medium containing 1 mg/ml of G418. Two weeks later, G418-resistant colonies were recorded. The titer of undiluted  $\alpha$ D virus stock was 10<sup>7</sup> G418<sup>r</sup> col/ml.

For infection, 1 million ANA-1 cells were incubated with 1 ml of  $\alpha$ D or N2 viral supernatants for 24 h in the presence of 5  $\mu$ g/ml of polybrene. At the end of infection, the spent supernatants were replaced with 5 ml of fresh D10F containing 1 mg/ml of G418. After 2 wk, the resistant cells were then expanded and passaged in D10F without G418.

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<sup>3</sup> Abbreviations used in this paper: NO, nitric oxide; PTK, protein tyrosine kinases; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; NMMA, N<sup>G</sup>-monomethyl-L-arginine; D10F, DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 10  $\mu$ g/ml gentamicin.

<sup>4</sup> R. Daniel, P. M. C. Wong, A. D. Kang, E. Moran, M. S. Moore, and S. W. Chung. Specific association of type I c-abl with Ran/TC4 GTPase and type IV c-abl with cdc2 and p53. *Submitted for publication*.

### Growth curve analysis

In all, 500,000  $\alpha$ D-, N2-transduced, or untransduced ANA-1 cells were plated in 1 ml of D10F in each well of a 24-well plate and cultured for 24, 48, and 72 h. For each time point, three independent samples were set up. Cell viability was evaluated by trypan blue (Sigma, St. Louis, MO) exclusion.

### LPS stimulation

Because both endotoxin protein and protein-free LPS are found to be potent activators of the mouse immune system by acting as B cell mitogens and polyclonal activators of B cells (26, 27), the LPS used in this study was purified. Two of the most extensively used protocols for extracting the endotoxin from Gram-negative bacteria are the Boivin method and the Westphal method (28). Protein-free LPS purified from *Salmonella typhimurium* by the phenol-water extraction method was used in all experiments described in this study (20, 29, 30).

A total of 500,000  $\alpha$ D-, N2-transduced, or untransduced ANA-1 cells were plated in 1 ml of D10F in each well of a 24-well plate in the presence of 10  $\mu$ g/ml of protein-free purified LPS, and the cells were cultured for 24, 48, 72, and 96 h. The cell number in each of three replicates at each time point was recorded and the cell viability was evaluated by trypan blue staining. Cell-free supernatants from these samples were also collected for NO and TNF- $\alpha$  assay, and the cells were harvested for DNA fragmentation analysis.

To examine the effects of iNOS (inducible nitric oxide synthase) inhibitor on NO production of  $\alpha$ D- or N2-transduced or untransduced ANA-1 cells stimulated with 10  $\mu$ g/ml of LPS, we added NMMA ( $N^G$ -monomethyl-L-arginine; Calbiochem-Behring, La Jolla, CA; catalog no. 475856) into the culture medium at a concentration of 0.2 mM (31, 32). The cell culture was incubated for 72 h. NO production of cellfree supernatants was then measured.

### Immunoprecipitation and Western blot analysis

Ten million  $\alpha$ D-, N2-transduced, or untransduced ANA-1 cells were cultured in 20 ml of D10F for 48 h with or without 10  $\mu$ g/ml of LPS. Five million cells from each sample were harvested, washed twice with PBS, and lysed in 500  $\mu$ l of lysis buffer containing 10 mM Tris-HCl (pH 8.0), 130 mM NaCl, 1% Triton X-100, 5 mM EDTA, protease inhibitors (10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM aminoethyl benzene sulfonyl fluoride), and phosphatase inhibitor (400  $\mu$ M sodium vanadate). After 30 min of incubation on ice, the lysates were centrifuged at 16,000  $\times$  g for 15 min at 4°C. The supernatants were collected and transferred into siliconized tubes, and the monoclonal anti-Abl Ab 8E9 (a gift from Dr. Jean Y. J. Wang, University of California at San Diego) was added to a final concentration of 20  $\mu$ g/ml. Immune complexes were allowed to form during an overnight rotation at 4°C. Afterward, 30  $\mu$ l of protein G-agarose (Boehringer Mannheim, Indianapolis, IN) was added, and the tubes were rocked at 4°C for 2 h. The Ag-Ab-protein G-agarose complexes were washed three times with lysis buffer. The pellets were resuspended with 40  $\mu$ l of 2 $\times$  sample buffer containing 100 mM Tris-HCl (pH 6.8), 40% (v/v) glycerol, 2% SDS, 0.02% bromophenol blue, and 2% 2-ME, boiled for 5 min, and spun at 16,000  $\times$  g for 5 min at 4°C. The supernatants were then collected and separated by SDS-PAGE. Biotinylated protein standards (Bio-Rad, Richmond, CA) for SDS-PAGE were used as m.w. markers.

Electrophoresis was conducted in the presence of Tris-glycine buffer with a Tall Mighty Small vertical slab gel unit (Hoefer, San Francisco, CA). After electrophoresis, the gel was soaked in Tris-glycine transfer buffer containing 20% (v/v) methanol for 30 min and the proteins were transferred in the same buffer onto the Immobilon-P Transfer Membrane (Millipore, Bedford, MA). The membrane was blocked with blocking solution (Life Technologies, Grand Island, NY) for 1 h at room temperature, and blotted with 3  $\mu$ g/ml of 8E9 Ab in blocking solution for another 1 h at room temperature. The membrane was then washed twice for 5 min each time with a washing solution consisting of 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20. Next, it was incubated with 1:2000 biotinylated goat anti-mouse IgG in blocking solution (Life Technologies) for 30 min at room temperature, washed twice with the Tris-buffered saline with Tween 20 washing solution, incubated further with 1:2500 streptavidin-alkaline phosphatase conjugate (Life Technologies) for 30 min at room temperature, and followed by another four washes. The proteins were visualized by using a chemiluminescent substrate (Boehringer Mannheim) and the gel exposed to an x-ray film. In some experiments, separate aliquots of protein lysates were stained with Coomassie blue after electrophoresis. Protein lysates of 2  $\times$  10<sup>5</sup> cells per sample were separated on a 7% SDS-PAGE. The gel was stained with 0.05% Coomassie brilliant blue

(Boehringer Mannheim) for 30 min at room temperature. It was then washed in 5% ethanol/7% acetic acid for 4 h at room temperature.

For iNOS Western blot analysis, the  $\alpha$ D-, N2-transduced, or untransduced ANA-1 cells were incubated for 72 h with or without 10  $\mu$ g/ml of LPS. Cell lysates of 1  $\times$  10<sup>6</sup> cells were resolved on a 7% SDS-PAGE, transferred to Immobilon-P Transfer Membrane, and blotted with 1  $\mu$ g/ml of affinity-purified rabbit polyclonal iNOS Ab (Santa Cruz Biotechnology, Santa Cruz, CA, catalog no. SC-650) (33–36). Next, the membrane was incubated with 1:5000 anti-rabbit IgG-alkaline phosphatase conjugate (Santa Cruz Biotechnology, catalog no. SC-2007). The 130-kDa iNOS protein bands (34, 37) were visualized by using a chemiluminescence system (Boehringer Mannheim).

### Measurement of NO

The cellfree supernatants were collected at the end of cell culture. The concentration of nitrite (NO<sub>2</sub><sup>-</sup>) was measured by colorimetric Griess reaction and used as an indicator of NO production (38). In a 96-well plate, 100- $\mu$ l aliquots of culture supernatants or standard solutions (NaVO<sub>2</sub>; Sigma) were mixed with equal volumes of the Griess reagent (2% phosphoric acid, 1% sulfanilamide, 0.1% naphthylethylene-diamine dihydrochloride; Sigma). Color change began almost immediately. Ten minutes after initiation of reaction at room temperature, absorbance was measured using an automated microplate reader (Bio-Tek Instruments) at 550 nm. The concentration of NO<sub>2</sub><sup>-</sup> was calculated and quantitated by comparing it with the color developed when NaVO<sub>2</sub> standards were used.

### Determination of TNF- $\alpha$ concentration

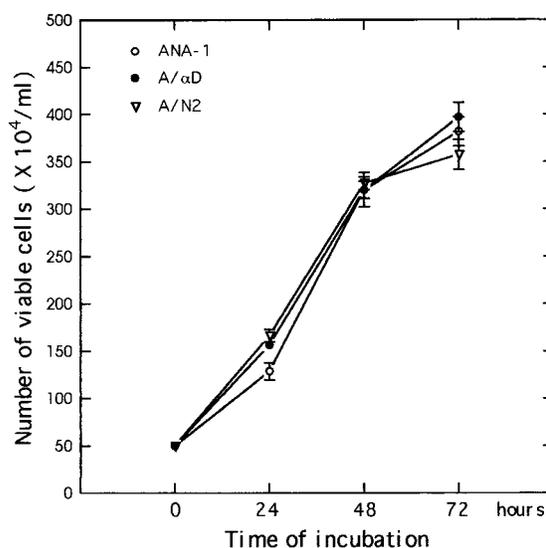
Cells were incubated with or without LPS for 72 h and supernatants were collected for TNF- $\alpha$  assay. The TNF- $\alpha$  concentrations were determined immediately by using an Enzyme Immunoassay kit (Immunotech, Marseille, France; catalog no. 1121) (39, 40).

### DNA fragmentation analysis

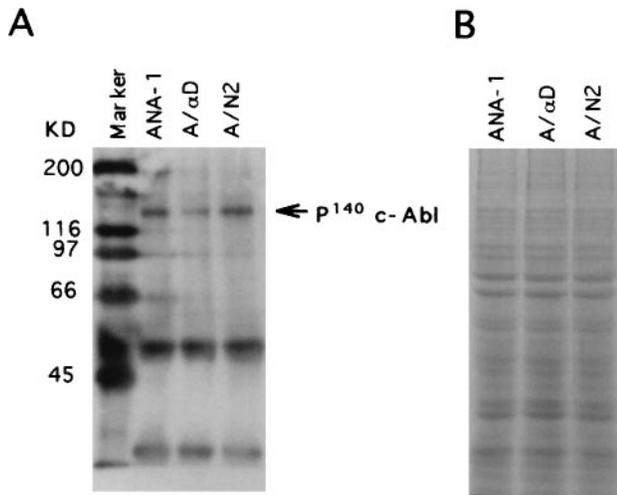
Genomic DNA of various cells, with or without LPS stimulation, were extracted by the phenol/chloroform method (23). A total of 5  $\mu$ g of genomic DNA of each sample was then run on a 2% agarose gel at 40 V for 2 h.

### C-Abl kinase assay

Cells (2  $\times$  10<sup>7</sup> per sample) were stimulated with 5  $\mu$ g/ml of LPS for 48 h. Then, cells were lysed in a lysis buffer containing 10 mM Tris-Cl, pH 7.6,



**FIGURE 1.** Growth curves of  $\alpha$ D- or N2-transduced and untransduced ANA-1 cells. The  $\alpha$ D retrovirus vector carries anti-c-abl antisense sequence and a *neo<sup>r</sup>* gene. ANA-1 cells were infected with  $\alpha$ D virus or N2 control virus and selected by 1 mg/ml of G418. Transduced or untransduced ANA-1 cells were plated into each well of a 24-well plate at 5  $\times$  10<sup>5</sup>/ml. The number of viable cells was enumerated every 24 h. The results were mean  $\pm$  SD in a representative experiment performed in triplicate. Four separate experiments were performed, and similar results were obtained. ANA-1, untransduced ANA-1 cells; A/ $\alpha$ D,  $\alpha$ D-transduced ANA-1 cells; A/N2, N2-transduced ANA-1 cells.



**FIGURE 2.** Reduction of c-Abl level in  $\alpha$ D-transduced ANA-1 cells. *A*, Cell lysates of  $5 \times 10^6$  cells of  $\alpha$ D- or N2-transduced and untransduced ANA-1 cells were immunoprecipitated with anti-Abl Ab (8E9) and incubated with protein G-agarose. The complexes were collected, washed, and boiled. The supernatant was resolved on a 7% SDS-PAGE, transferred to an Immobilon-P membrane, and blotted with 8E9 Ab. The c-Abl bands were visualized by using a chemiluminescence system. Marker, high m.w. marker. *B*, The protein lysates of  $2 \times 10^5$  cells of ANA-1, A/ $\alpha$ D, or A/N2 were separated on a 7% SDS-PAGE. The gel was then stained with Coomassie blue staining solution.

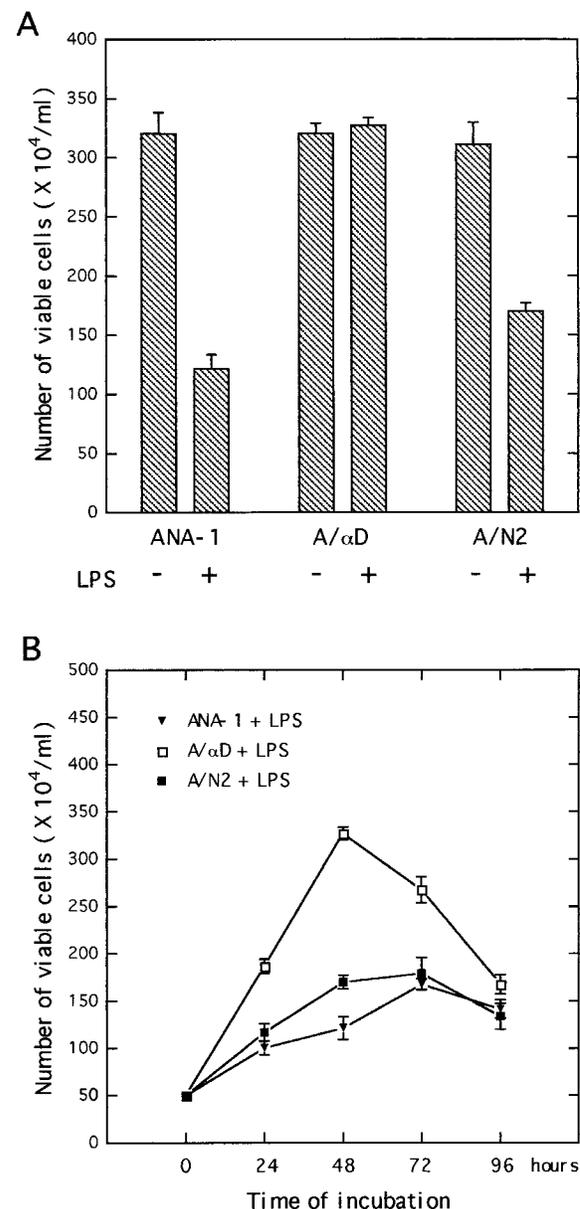
5 mM EDTA, 130 mM NaCl, 1% Triton X-100, and protease inhibitors. Lysates of  $1 \times 10^7$  cells per sample were immunoprecipitated with 8E9 c-Abl Ab. Protein G-agarose (Boehringer Mannheim) was used to pull down the protein-Ab complexes. A kinase assay was performed in 50 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 20  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP, and protease inhibitors at 30°C for 30 min. The kinase products were resolved on 7% SDS-PAGE, gel dried, and exposed to x-ray film. To determine the c-Abl level,  $1 \times 10^7$  cells per sample were lysed in the lysis buffer (see above), immunoprecipitated with 8E9 Ab, and the immunoprecipitates resolved on 7% SDS-PAGE. The proteins were then transferred onto the Immobilon-P Transfer Membrane (Millipore), which was blotted with 8E9 Ab. The proteins were visualized using a chemiluminescent detection system (Life Technologies).

## Results

### *ANA-1 cells expressing reduced c-Abl have the same growth rate as controls*

ANA-1 macrophages were transduced with the  $\alpha$ D vector capable of expressing anti-c-abl antisense RNA (23) and neomycin resistance. Positively transduced cells were then selected by virtue of G418 resistance, pooled, and expanded. As a control, pooled ANA-1 cells positively transduced with the N2 vector carrying the neo<sup>r</sup> gene (24) were also similarly established. To determine whether there was any difference between these cell lines in terms of cell proliferation, we did growth curve analysis on the parental ANA-1 cells,  $\alpha$ D-transduced ANA-1 (A/ $\alpha$ D) cells, and N2-transduced ANA-1 (A/N2) cells. Figure 1 shows that no significant difference between these lines could be observed.

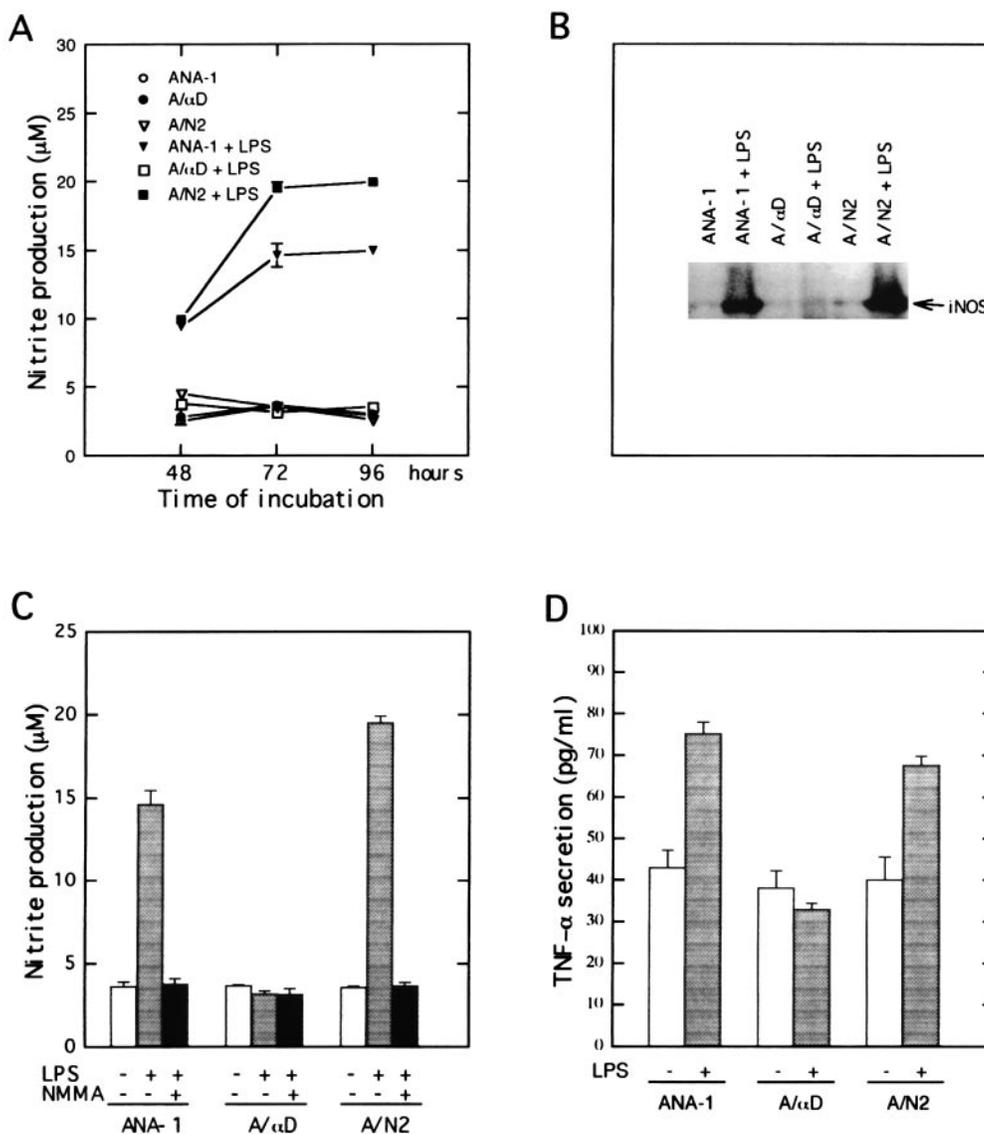
To verify that A/ $\alpha$ D cells, as opposed to control cells, express reduced c-Abl, we performed immunoprecipitation and a Western blot on lysates of these cell lines using the monoclonal anti-c-Abl 8E9 Ab. Lysates from 5 million cells of each line were immunoprecipitated with 8E9, followed by SDS-PAGE and Western blot analysis using 8E9 also as the blotting Ab. The intensity of the p140 c-Abl band in A/ $\alpha$ D was five times lower than that of parental ANA-1 cells and A/N2 cells, as measured by densitometer



**FIGURE 3.** Growth of  $\alpha$ D- or N2-transduced and untransduced ANA-1 cells with LPS stimulation. *A*,  $\alpha$ D- or N2-transduced and untransduced ANA-1 cells were plated into a 24-well plate at  $5 \times 10^5$ /ml, and incubated with or without 10  $\mu$ g/ml of LPS. The number of viable cells was recorded after 48 h. The data presented are the mean  $\pm$  SD of a representative experiment performed in triplicate. The growth of  $\alpha$ D-transduced cells was not inhibited by LPS stimulation, and the results from four individual repeated experiments were similar. *B*, Growth curves of  $\alpha$ D- or N2-transduced and untransduced ANA-1 cells with LPS stimulation. The cells were set up and stimulated with LPS as in *A*, and then the number of viable cells was counted every 24 h. The results are expressed as mean  $\pm$  SD of a representative experiment performed in triplicate. The results of four individual repeated experiments were similar. After LPS stimulation, the proliferation of N2-transduced and untransduced ANA-1 cells was inhibited.  $\alpha$ D-transduced ANA-1 cells continued to proliferate when incubated with LPS for 24 and 48 h, and then cell growth was inhibited when incubated for 72 and 96 h. The growth rate of  $\alpha$ D-transduced cells with LPS stimulation is significantly higher than that of N2-transduced or untransduced ANA-1 cells with LPS stimulation.

(Fig. 2A). The total amount of protein used per sample was the same (Fig. 2B).

A similar level of c-Abl reduction was observed in A/ $\alpha$ D cells stimulated with LPS compared with that of LPS-treated control



**FIGURE 4.** NO and TNF- $\alpha$  production of  $\alpha$ D- or N2-transduced ANA-1 cells, with or without LPS stimulation. *A*, NO production.  $\alpha$ D- or N2-transduced and untransduced ANA-1 cells were plated into a 24-well plate at  $5 \times 10^5$ /ml, and incubated with or without 10  $\mu$ g/ml of LPS for 48, 72, and 96 h. The culture supernatants were collected at the end of the incubation. The concentrations of nitrite ( $\text{NO}_2^-$ ) were used as an indicator of NO production and were measured by the Griess reaction, with sodium nitrite as the standard. Data presented are the means  $\pm$  SD of two determinations from one representative experiment of three. *B*, Western blot of iNOS gene expression.  $\alpha$ D- or N2-transduced and untransduced ANA-1 cells were incubated for 72 h with or without 10  $\mu$ g/ml of LPS, and lysates of  $1 \times 10^6$  cells were resolved on a 7% SDS-PAGE, and blotted with iNOS Ab. The 130-kDa iNOS protein bands were visualized by using a chemiluminescence system. Data are from one of two similar experiments. *C*, Influence of iNOS inhibitor (NMMA) on NO production. Cells were incubated for 72 h with or without 10  $\mu$ g/ml of LPS in the absence or presence of 0.2 mM of NMMA. The NO production was measured as in *A*. Data presented are the means  $\pm$  SD of two determinations from one representative experiment of three. *D*, TNF- $\alpha$  secretion. Cells were incubated for 72 h with or without LPS as in *A*. The TNF- $\alpha$  concentrations of culture supernatants were determined with an Enzyme Immunoassay kit. Results are expressed in pg/ml of TNF- $\alpha$  secreted and are from one representative experiment of three.

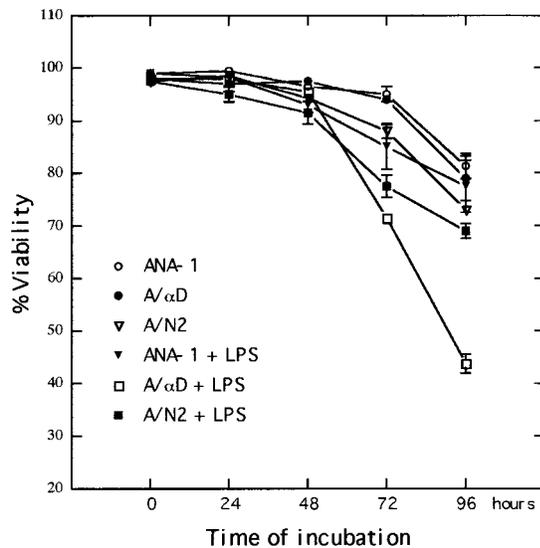
cells (data not shown). The P<sup>140</sup> c-Abl level was reduced in  $\alpha$ D-transduced ANA-1 cells after 48 h of LPS stimulation. The amount of c-Abl in each band on the Western blot gel was established by densitometry analysis by the MacBAS computer program. The relative intensity ratios of c-Abl bands of the untransduced,  $\alpha$ D-, or N2-transduced ANA-1 cells were 0.87, 0.32, and 1, respectively. These data indicate that A/ $\alpha$ D cells expressed reduced c-abl, but their growth rate appeared to be the same as that of the controls.

*LPS-stimulated A/ $\alpha$ D cells were not growth inhibited and did not have enhanced NO and TNF- $\alpha$  production*

ANA-1 macrophages are responsive to LPS (41). To examine the effect of reduced c-Abl in these cells, we studied  $\alpha$ D-, N2-transduced,

and untransduced ANA-1 cells after LPS stimulation. ANA-1 and A/N2 cells responded to LPS by undergoing growth inhibition (Fig. 3), which was dose and time dependent. This growth inhibition could be observed at 1  $\mu$ g/ml, 10  $\mu$ g/ml, and 100  $\mu$ g/ml of LPS and at 24 to 96 h after stimulation. By contrast, A/ $\alpha$ D cells did not undergo growth inhibition when stimulated with LPS. Instead, they continued to proliferate within the first 48 h (Fig. 3).

Related to the growth inhibition after LPS stimulation was the activation of ANA-1 cells. This is shown by the ability of these LPS-stimulated macrophages to produce NO, which is controlled by nitric oxide synthase (NOS) (42). Measurement of NO production in cell culture supernatants was determined by the Griess reaction (38). Without LPS stimulation, NO production by all



**FIGURE 5.** Viability of  $\alpha$ D- or N2-transduced ANA-1 cells with or without LPS stimulation.  $\alpha$ D- or N2-transduced and untransduced ANA-1 cells were plated into a 24-well plate at  $5 \times 10^5$ /ml, and incubated with or without  $10 \mu\text{g/ml}$  of LPS. The cell viability was evaluated by trypan blue exclusion dye. The number of viable and dead cells was recorded every 24 h. The results are expressed as the mean  $\pm$  SD of a representative experiment performed in triplicate. The results of four individual repeated experiments were similar. After LPS stimulation for 96 h, the viability of  $\alpha$ D-transduced ANA-1 cells was lower.

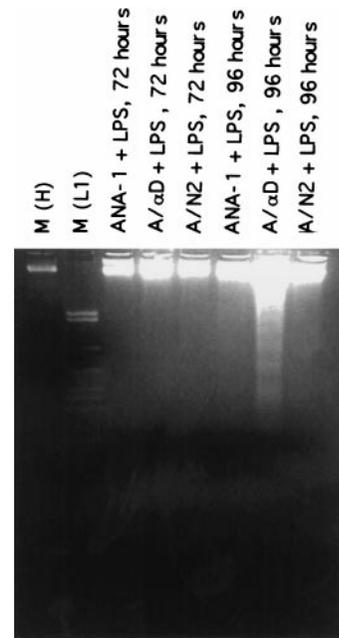
ANA-1 cells, transduced or untransduced, was minimal (Fig. 4A). After LPS stimulation, NO production was obvious in parental ANA-1 cells and A/N2 cells but not in A/ $\alpha$ D cells (Fig. 4A).

Next, we examined expression of other genes associated with macrophage activation. By Western blot analysis, we showed that iNOS protein production was significantly increased in LPS-stimulated parental ANA-1 cells and A/N2 cells but not in A/ $\alpha$ D cells (Fig. 4B). The NOS inhibitor, NMMA, significantly inhibited NO production by LPS-stimulated parental ANA-1 cells and A/N2 cells (Fig. 4C). We further showed that TNF- $\alpha$  was secreted by LPS-stimulated parental ANA-1 cells and A/N2 cells, but not A/ $\alpha$ D cells (Fig. 4D). Thus, multiple LPS-induced gene expression patterns were affected as a result of reduced c-Abl in stimulated macrophages.

#### LPS-stimulated A/ $\alpha$ D cells proliferated initially and then underwent apoptosis

As indicated in Figure 3, after LPS stimulation the A/ $\alpha$ D cells but not the control cells continued to grow. However, upon longer incubation, LPS-stimulated A/ $\alpha$ D had more pronounced cell death compared with A/N2 or parental ANA-1 cells. At 96 h after LPS stimulation, the percentage of viability of A/ $\alpha$ D cells was 40%, whereas for A/N2 and ANA-1 cells, it remained higher than 70% (Fig. 5), even though the growth of these cells was inhibited by LPS (Fig. 3B).

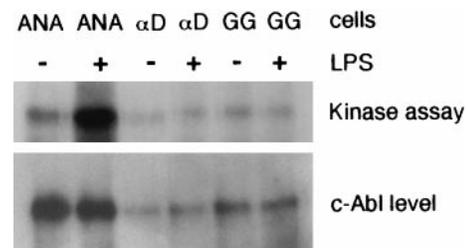
Decreased viability of A/ $\alpha$ D cells after LPS stimulation suggests induction of apoptosis. To examine this, we extracted genomic DNA from these cells and fractionated them in a 2% agarose gel, followed by staining the gel with ethidium bromide. As indicated in Figure 6, a significant amount of DNA fragmentation occurred in A/ $\alpha$ D cells stimulated with LPS for 96 h but not 72 h, whereas neither A/N2 nor parental ANA-1 cells revealed DNA fragmentation.



**FIGURE 6.** DNA fragmentation analysis. A total of  $2 \times 10^6$  of  $\alpha$ D- or N2-transduced and untransduced ANA-1 cells were plated in 4 ml of D10F medium in each well of a six-well plate, incubated with or without  $10 \mu\text{g/ml}$  of LPS for 72 or 96 h. The genomic DNA was extracted by the phenol/chloroform method. In all,  $5 \mu\text{g}$  of genomic DNA of each sample was then run on a 2% agarose gel. The experiments were repeated three times and the results were about the same. DNA fragmentation occurred clearly in the  $\alpha$ D-transduced ANA-1 cells after LPS stimulation for 96 h. M(H),  $\lambda$ DNA/*Hind*III marker; M(L1), pSVLCAT/*Hinf*I m.w. marker.

#### Reduced c-Abl kinase activity after LPS stimulation in $\alpha$ D-transduced ANA-1 cells and GG2EE cells

Recently, we have shown that type I c-Abl kinase activity is enhanced in lymphoid cells as a result of LPS stimulation (Ref. 21).<sup>4</sup> Based on the results shown in Figure 2, in which the c-Abl level in ANA-1 cells is reduced,  $\alpha$ D-transduced and LPS-stimulated ANA-1 cells should have reduced c-Abl kinase activity. We therefore proceeded to perform a kinase assay after immunoprecipitation with anti-c-Abl Ab. Indeed, Figure 7 shows that enhancement of c-Abl kinase activity was absent in  $\alpha$ D-transduced and LPS-stimulated ANA-1 cells. We further showed an absence of up-regulation of c-Abl kinase activity in LPS-stimulated GG2EE macrophage cells (Fig. 7), which were derived from LPS-hyporesponsive C3H/HeJ mice (43) and contain a mutated LPS-responsive gene encoding for Ran/TC4 (20). Reduced c-Abl kinase



**FIGURE 7.** C-Abl kinase is activated by LPS stimulation in ANA-1 cells, but not in GG2EE cells. ANA-1,  $\alpha$ D-transduced ANA-1, and GG2EE cells were stimulated with LPS for 48 h. After stimulation, cells were lysed, and c-Abl was immunoprecipitated and subjected to kinase assay. The c-Abl kinase activity was evaluated by autophosphorylation of the c-Abl protein. ANA, untransduced ANA-1 cells;  $\alpha$ D,  $\alpha$ D-transduced ANA-1 cells; GG, GG2EE macrophage cells.

activity in GG2EE cells is significant because we have also shown recently that Ran physically associates with type I c-Abl, accounting for c-Abl-enhanced kinase activity.<sup>4</sup> This reduced activity may be related to reduced complex formation. Indeed, when we performed immunoprecipitation using anti-Ran Ab followed by measuring the kinase activity of c-Abl, we noticed a decline in the level of Ran/type I c-Abl complex, as well as the activity of c-Abl kinase in GG2EE cells stimulated with LPS compared with the controls (data not shown).

## Discussion

In this study, we have shown that reduction of c-Abl in ANA-1 macrophages resulted in no change in cell growth but impairment in their activation by LPS to produce NO. These data suggest that c-Abl is involved in signal transduction pathways for LPS-mediated macrophage activation. The molecular mechanisms of intracellular signaling have been shown to involve certain PTK (12–19). The induction of tumoricidal properties, arachidonic acid, and TNF secretion by LPS-stimulated macrophages can be blocked by PTK inhibitors (12–19). Thus, the involvement of c-Abl tyrosine kinase in macrophage activation by LPS is consistent with the findings of these investigations.

ANA-1 macrophages responded to LPS by undergoing growth arrest, followed by activation in terms of NO production, iNOS expression, and TNF production (Figs. 3 and 4). By contrast, ANA-1 cells expressing a reduced level of c-Abl continued to proliferate in the presence of LPS, but at 72 h after stimulation they started to undergo apoptosis (Figs. 5 and 6). NO has been shown to have an antiproliferative effect (44). Thus, the proliferation of ANA-1/ $\alpha$ D cells may be a direct consequence of the reduced NO production. Our data also suggest the following. 1) LPS stimulated ANA-1 macrophages to initiate signaling events for NO production. Once triggered by LPS, the process appears to be irreversible. 2) c-abl participates as an intermediate in this signaling pathway but is downstream from the initial event of LPS stimulation, as reduction of c-Abl did not inhibit the process initiated by LPS. 3) As a result of blocking the activation of ANA-1/ $\alpha$ D cells, an alternative signaling pathway for apoptosis takes place. The mechanism of c-Abl involvement in NO, iNOS, and TNF production by LPS-activated macrophages is unclear at present. One possibility is that c-Abl may affect iNOS expression via a mechanism not related to LPS at all. We consider this possibility unlikely because we have recently shown an LPS-dependent complex formation between type I c-Abl and Ran/TC4.<sup>4</sup> The latter has been shown to be an LPS-responsive gene (20).

We have recently shown that type IV c-abl is inhibitory to apoptosis, and type I c-abl is necessary for LPS-induced differentiation in lymphoid cells (21). Involvement of type IV c-abl is probably achieved by indirect activation of p53,<sup>4</sup> which is known to play an important role in the induction of apoptosis. In this study, induction of the apoptosis pathway in ANA-1/ $\alpha$ D macrophages may or may not be directly associated with the inhibition of LPS-mediated activation as a result of reduced c-abl. Reduction of c-abl was achieved by antisense RNA, and the vector design of the target c-abl sequence does not discriminate between the predominant type I or type IV c-abl isoforms. Clearly, antisense treatment resulted in only a fivefold reduction of c-Abl. The level of c-Abl, however, appears to be sensitive in regulating various aspects of cell growth (45–48). For example, overexpression of c-Abl has been shown to induce growth arrest (49, 50), while reduction of c-Abl leads to deregulation in cell cycle (51) and apoptosis (21, 52).<sup>4</sup>

If type IV c-abl is inhibitory to apoptosis and if it is reduced in ANA-1/ $\alpha$ D cells, one might expect to see apoptosis even in LPS-unstimulated cells. This was not the case because without LPS stimulation ANA-1/ $\alpha$ D cells grew at the same rate as ANA-1/N2 cells or untransduced cells (Fig. 1). We have also shown recently that c-abl-mediated apoptosis in myeloid cells but not in lymphoid cells can be rescued by growth factors (23). Since ANA-1 cells are myeloid cells, unstimulated ANA-1/ $\alpha$ D cells not undergoing apoptosis also may have been rescued by growth factors. However, once LPS stimulation is initiated in ANA-1/ $\alpha$ D cells, the ability to be rescued by growth factors may be abolished. Alternatively, another apoptotic pathway associated with the blocking of LPS-induced signal transduction may be involved when the cells are stimulated with the mitogen.

By establishing a functional cloning strategy, we have recently shown that Ran/TC4 is an LPS-responsive gene (20). Molecular cloning and sequencing of the Ran/TC4 cDNA from cells of LPS-hyporesponsive C3H/HeJ mice indicated the existence of a specific point mutation at the 3' untranslated region of the cDNA.<sup>5</sup> Introduction of the wild-type but not this mutant Ran/TC4 cDNA into primary B cells of C3H/HeJ mice corrected the deficiency.<sup>5</sup> Most interestingly, we showed that type I c-Abl forms a complex with Ran/TC4 in response to LPS stimulation, and the level of this complex is positively correlated with the LPS dose.<sup>4</sup> Therefore, it will be of interest to see whether Ran/TC4 is also involved in NO production by LPS-activated macrophages.

The mechanism by which macrophages recognize LPS is not well understood. Although the glycosyl-phosphatidylinositol-linked membrane protein CD14 plays a role by binding directly to a complex between LPS and the LPS-binding protein present in serum (53), other cell surface LPS receptors probably exist. At this point, we have no evidence either way as to whether the signal pathway we have found is CD14 dependent or independent. This awaits further studies.

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