Biochemical and Biophysical Research Communications **271,** 70–74 (2000)
doi:10.1006/bbrc.2000.2594, available online at http://www.idealibrary.com on IDEAL®

IgG-Coated Erythrocytes Augment LPS-Stimulated TNF-α Secretion, TNF-α mRNA Levels, and TNF-α mRNA Stability in Macrophages

Craig A. H. Richard,[1] Brian D. Wilcox, and Daniel J. Loegering[2]
Center for Cardiovascular Sciences, Albany Medical College, Albany, New York 12208

Received March 14, 2000

Previous studies have shown that IgG-coated erythrocytes (EIgG) augment the LPS-stimulated increase in serum TNF-α levels in animals and the LPS-stimulated secretion of TNF-α by isolated macrophages. The present study evaluated the mechanism for the effect of EIgG on LPS-stimulated TNF-α secretion in the murine macrophage cell line, RAW 264.7. Incubation of the macrophages with EIgG or IgG-coated glass beads caused a dose-dependent augmentation of LPS-stimulated TNF-α secretion. The addition of EIgG increased the rate of LPS-stimulated TNF-α protein secretion between 2 and 4 hr after LPS. Accordingly, EIgG increased the levels of TNF-α mRNA at 2 and 3 hr after LPS. The increase in the LPS-stimulated TNF-α mRNA levels caused by EIgG was associated with an increase in TNF-α mRNA stability. Thus, the augmentation of LPS-stimulated TNF-α secretion by EIgG was associated with an increase in TNF-α mRNA levels which at least partly resulted from an increase in the stability of TNF-α mRNA.

Tumor necrosis factor alpha (TNF-α) contributes to the normal proliferation, differentiation, and function of many cell types (1). In addition to its beneficial effects, TNF-α plays a role in several pathological processes. When produced in excess, TNF-α can cause or exacerbate disseminated intravascular coagulation, adult respiratory distress syndrome, liver failure, acute renal tubular necrosis, acute pancreatitis, chronic heart failure and gastrointestinal necrosis (2). In seriously injured patients, these effects contribute to the development of multiple organ dysfunction which is associated with a high mortality (2). TNF-α is also important in the pathogenesis of rheumatoid arthritis as indicated by the remarkable therapeutic effect of agents that neutralize the actions of TNF-α (3). Therefore, a thorough understanding of factors that influence the production of TNF-α will provide a rational basis for the development of additional treatments for TNF-α-mediated pathologies.

Lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria is a potent stimulus for TNF-α secretion by macrophages (4, 5). While LPS alone is capable of rapidly stimulating a high rate of TNF-α secretion, factors such as interferon-γ, granulocyte macrophage-colony stimulating factor and macrophage-colony stimulating factor are capable of priming macrophages for an augmented TNF-α response to LPS (6–8). The augmentation of TNF-α secretion caused by interferon-γ was associated with an increase in both TNF-α mRNA stability and the rate of TNF-α gene transcription (9).

Previous studies from this laboratory have shown that the prior injection of IgG-coated erythrocytes (EIgG) can augment the LPS-stimulated increase in serum TNF-α levels in rats and that the phagocytosis of EIgG by isolated macrophages increases LPS-stimulated TNF-α secretion (10). While it is well established that signaling via Fcγ receptors (FcγR) is capable of stimulating TNF-α secretion by itself (11–13), this was the first study to show that FcγR signaling can prime for LPS-stimulated TNF-α secretion. The present study further characterized the effect of EIgG on LPS-stimulated TNF-α secretion by the murine macrophage cell line, RAW 264.7. The augmented TNF-α secretion caused by EIgG was associated with an increase in TNF-α mRNA levels and an increase in the stability of TNF-α mRNA.

METHODS

Macrophages. RAW 264.7 cells, a murine macrophage-like cell line, were maintained and utilized for experiments in Roswell Park

[1] Current address: Department of Obstetrics, Gynecology and Reproductive Science, Lab 420, Magee Women's Research Institute, University of Pittsburgh, Pittsburgh PA 15213.
[2] To whom correspondence should be addressed at Center for Cardiovascular Sciences, MC 8, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208. Fax: 518-262-8101. E-mail: loegerd@mail.amc.edu.

0006-291X/00 $35.00

Memorial Institute media (RPMI) supplemented with 10% heat-inactivated calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were allowed to adhere overnight in 48 well plates (1.5×10^5/well) or 6 well plates (2.5×10^6/well) and were washed 2 h prior to the experiment.

Preparation of IgG-coated particles. Erythrocytes coated with IgG (EIgG) were prepared by incubating washed sheep erythrocytes (1×10^9/ml) with a 1:50 dilution of anti-sheep erythrocyte IgG (Accurate, Westbury, NY) at 37°C for 1 h (14). EIgG were washed 3× in PBS prior to use. Phagocytosis of EIgG was quantified by using ^{51}Cr-labeled EIgG and determining the amount of radioactivity associated with the macrophages after hypotonic lysis of non-ingested EIgG (14).

Glass beads coated with IgG (BIgG) were prepared by sequentially coating beads (2.5 μm in diameter, Duke Scientific, Palo Alto, CA) with albumin and then anti-albumin IgG as previously described (15). The phagocytosis of BIgG was determined microscopically. The total number of BIgG associated with the macrophages was counted under phase contrast and noningested BIgG were identified by the ability to bind FITC-labeled goat anti-rabbit IgG. Beads coated with only albumin were used as a negative control.

Experimental protocol. LPS-stimulated TNF-α secretion or mRNA levels were determined after incubation of the macrophages with EIgG. Different doses of EIgG (0.1, 1.0 or 3×10^6/ml), different doses of LPS (0.1, 0.3, 1.0, 3.0 or 100 ng/ml) and different times between EIgG and LPS (0, 2, 6, or 18 h) were tested. The time course of LPS-stimulated TNF-α secretion and mRNA levels was determined from measurements at 1, 2, 3, 4, 5, or 8 h after LPS. Controls included PBS (EIgG diluent), sheep erythrocytes (E) not coated with IgG or the amount of anti-erythrocyte IgG used to prepare EIgG.

TNF-α ELISA. TNF-α protein was determined as previously described (10). Monoclonal hamster anti-murine TNF-α and the polyclonal rabbit anti-murine TNF-α were obtained from R & D Systems (Minneapolis, MN), and the horseradish peroxidase-conjugated goat anti-rabbit IgG was from Southern Biotech (Birmingham, AL). Recombinant murine TNF-α (Genzyme, Cambridge, MA) was used for standards.

TNF-α mRNA. Total RNA was isolated from RAW 264.7 cells using a commercially available isolation reagent (ULTRA-spec.II; Biotex Labs, Edmonton, Alb, Canada) according to the manufacturer's protocol. Total RNA was electrophoresed in agarose gels and transferred overnight to nylon membranes (Bio-Rad Labs, Hercules, CA). cDNA probes were created by PCR and were based on the sequence determined by Caput *et al.* (16). Membranes were allowed to hybridize overnight with [^{32}P]dCTP random primer-labeled cDNA for TNF-α at 42°C. Membranes were washed 3×20 min in 2× SSC (1× = 0.15 M NaCl, 0.015 M Na citrate) at room temperature, 3×20 min in 1× SSC at 65°C, and analyzed on a phosphoimager (Storm 860, Molecular Dynamics, Sunnyvale, CA). Samples were normalized to methylene blue-stained 18s rRNA and data expressed as relative densitometry units (RDU).

TNF-α mRNA stability. The stability of TNFα mRNA was assessed by determining the rate at which the mRNA levels decrease after blocking transcription with 5,6 dichlorobenzimidazole riboside (DRB, Sigma, St. Louis, MO). DRB (100 μM) was added to the cells one hour after LPS and mRNA levels were determined at 0, 20, 40, 60, 90 and 120 min after DRB to establish the rate of TNF-α mRNA degradation (17).

RESULTS

EIgG augments LPS-stimulated TNF-α secretion. Incubation of RAW 264.7 cells with EIgG for 2 h before adding LPS resulted in a dose-dependent augmentation of LPS-stimulated TNF-α secretion (Fig. 1A). LPS alone stimulated the secretion of 742 ± 52 pg/ml

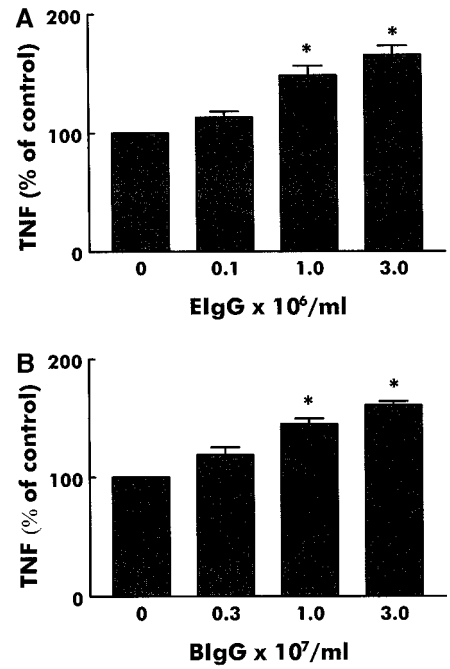

FIG. 1. Effect of EIgG and BIgG on LPS-stimulated TNF-α secretion. RAW 264.7 cells were incubated with the indicated number of IgG-coated erythrocytes (EIgG) (A) or IgG-coated glass beads (BIgG) (B) for 2 h, washed and LPS (100 ng/ml) added. Four hours after the addition of LPS, media was removed for analysis of TNF-α levels by ELISA. Values are expressed as the mean ± standard error of the mean for 3 experiments. *$P < 0.05$ compared with LPS only.

TNF-α. Unlike EIgG, erythrocytes not coated with IgG (E) or the amount of IgG used to prepare 1×10^7 EIgG (IgG), had no effect on the LPS-stimulated TNF-α secretion (Fig. 3A). EIgG without subsequent LPS caused the secretion of less than 5% of the amount of TNF-α elicited by LPS (Fig. 3A). BIgG caused a similar degree of augmentation as EIgG but a higher dose of BIgG was required (Fig. 1B). With the 3×10^6/ml dose of EIgG or BIgG, RAW 264.7 cells ingested about 3 EIgG or 6 BIgG per macrophage. Beads coated with only albumin were not phagocytosed and had no effect on LPS-stimulated TNF-α secretion (data not shown). With regard to the duration of the effect, the greatest augmentation of the LPS-stimulated TNF-α secretion was seen when the EIgG were added at the same time as LPS and the augmentation progressively decreased with time (Fig. 2). The relative augmentation of TNF-α secretion due to EIgG was similar for all doses of LPS that stimulated detectable TNF-α secretion (100, 3, 1, 0.3 ng/ml) (data not shown).

EIgG augments the LPS-stimulated increase in TNF-α mRNA. LPS caused an increase in TNF-α mRNA over the basal level observed in untreated cells (Fig. 3B). As with the TNF-α protein secretion, EIgG caused an augmentation of the LPS-stimulated TNF-α mRNA. Neither erythrocytes alone nor the amount of

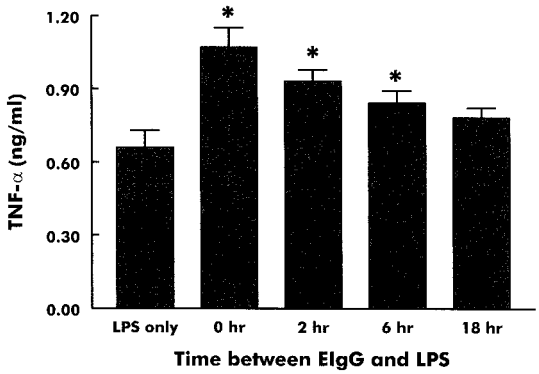

FIG. 2. Effect of adding LPS at different times after EIgG on TNF-α secretion. EIgG (3×10^6/ml) were added to RAW264.7 cells and then LPS (100 ng/ml) was added 0, 2, 6, or 18 h later. TNF-α levels in the media were determined at 4 h after LPS. Values are the mean $\pm$ standard error of the mean for 3 experiments. *$P < 0.05$ compared with LPS only.

IgG used to prepare EIgG augmented the LPS-stimulated TNF-α mRNA. EIgG alone caused a consistent but small increase in the TNF-α mRNA level.

Effect of EIgG on the time course of LPS-stimulated TNF-α mRNA and TNF-α secretion. EIgG increased the rate of LPS-stimulated TNF-α secretion between 2 and 4 h after LPS (Fig. 4A). The increase in TNF-α secretion was associated with an increase in the rate of LPS-stimulated TNF-α mRNA production between 1 and 2 after LPS (Fig. 4B). Thus, the increase in TNF-α mRNA corresponded to the times when secretion of TNF-α protein was augmented.

Effect of EIgG on TNF-α mRNA stability. TNF-α mRNA stability was assessed from the rate of degradation following the addition of DRB, an inhibitor of mRNA transcription. DRB was added 1 h after LPS. Under these conditions, LPS-stimulated TNF-α mRNA levels decreased with a half-time of 40 min (Fig. 5). With EIgG plus LPS, TNF-α mRNA was degraded at a slower rate (Fig. 5). Thus, the increased stability of TNF-α mRNA probably contributed to the augmented mRNA levels and TNF-α secretion caused by EIgG.

DISCUSSION

The present study characterized the EIgG augmentation of TNF-α secretion by LPS-stimulated macrophages. EIgG and BIgG caused a dose-dependent augmentation of LPS-stimulated TNF-α secretion. Maximum augmentation was seen when EIgG and LPS were added at the same time and the effect lasted for nearly 6 h. EIgG augmented TNF-α secretion for all doses of LPS that caused a detectable level of TNF-α secretion. The time course of LPS-stimulated TNF-α secretion revealed that EIgG increased the rate of TNF-α secretion at 2–4 h after LPS. The increase in TNF-α secretion due to EIgG was associated with an increase in TNF-α mRNA levels and an increase in mRNA stability.

Our studies on the ability of EIgG to prime for LPS-stimulated TNF-α secretion were based on the observation that injection of EIgG increased the mortality caused by LPS in rats (18, 19). The ability of EIgG to augment the TNF-α response to LPS suggests that the high levels of TNF-α may have contributed to the increased mortality due to LPS following the phagocytosis of EIgG (10). In contrast to the augmented TNF-α response to LPS, other studies have shown that the phagocytosis of EIgG phagocytosis is followed by a period of depressed macrophage phagocytic function and respiratory burst capacity (14, 20, 21).

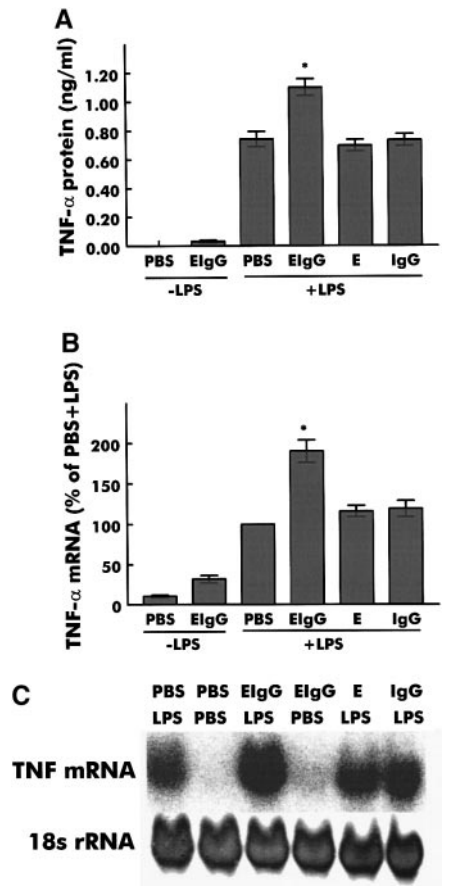

FIG. 3. Effect of EIgG on LPS-stimulated TNF-α protein secretion and TNF-α mRNA levels. RAW 264.7 cells were incubated with EIgG (3×10^6/ml), erythrocytes (E, 3×10^6/ml), or soluble IgG for 2 h, washed, and PBS or LPS (100 ng/ml) was added. For TNF-α protein secretion (A), media was removed 4 h after LPS addition and analyzed by ELISA. For mRNA levels (B), cells were lysed for RNA isolation 2 h after the addition of LPS. TNF-α mRNA was detected by Northern blot analysis and quantified using a phosphoimager. mRNA results are shown as the percent of the levels stimulated by LPS alone (PBS-LPS). A representative Northern blot is shown (C). Values are expressed as the mean $\pm$ the standard error of the mean for 3 experiments. *$P < 0.05$ compared with the PBS-LPS group.

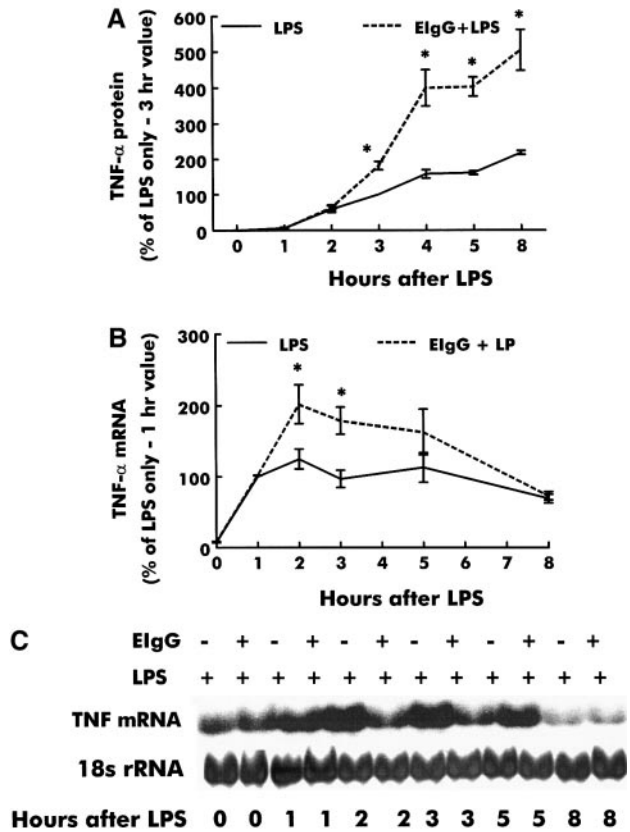

FIG. 4. Effect of EIgG on the time course of LPS-stimulated TNF-α protein secretion and mRNA levels. RAW 264.7 cells were incubated with EIgG (3×10^6/ml) plus LPS (100 ng/ml). At the indicated times after LPS, (A) media was removed for TNF-α protein analysis by ELISA or (B) cells were lysed for RNA isolation and determination of mRNA levels by Northern blot analysis. A representative Northern blot is shown (C). TNF-α protein values are expressed as the percent of the 3 h LPS only value and mRNA values are expressed as the percent of the 1 h LPS only value. Values are expressed as the mean $\pm$ the standard error of the mean for 3 experiments. $^*P < 0.05$ compared with the respective LPS value.

It is well established that the phagocytosis of EIgG or cross-linking FcγR can directly stimulate the secretion of cytokines such as TNF-α, IL-1β, IL-6, IL-8 and IL-10 by macrophages or monocytes (11, 13, 22–27). In the present study, the amount of TNF-α secretion stimulated by EIgG alone was less than 5% of that caused by LPS. Aside from our work, there are no previous studies on the ability of FcγR signaling to prime macrophages for LPS-stimulated TNF-α secretion but there are a few studies on other cytokines. Marsh *et al.* (28) demonstrated that monocytes adherent to an IgG-coated surface did not secrete detectable IL-1β but the LPS-stimulated IL-1β secretion doubled and the sensitivity to LPS increased 100-fold. Similarly, Selvan *et al.* (25) found a synergistic effect between immobilized IgG and LPS for the induction of I-309 mRNA in monocytes. Sutterwala *et al.* (29) have shown that LPS-stimulated IL-10 secretion was augmented by EIgG and that the IL-10 inhibited IL-12 secretion. This group also demonstrated that signaling via FcγR inhibited LPS-stimulated IL-12 secretion (30).

EIgG caused an increase in the rate of LPS-stimulated TNF-α secretion between 2 and 4 h after LPS. This timing corresponded to the effect of EIgG on LPS-stimulated TNF-α mRNA levels. These combined results suggest that the effect of EIgG on the increase in TNF-α protein secretion is due to an increase in TNF-α mRNA availability. This increase in TNF-α mRNA levels could have been caused by either an increase in the rate of TNF-α transcription or an increase in the stability of the TNF-α mRNA. Our results show that the increase in TNF-α mRNA levels was at least partially due to increased stability of the TNF-α mRNA. LPS-stimulated TNF-α mRNA, in the absence of EIgG, displayed a steady level of degradation with a half life of 40 min. In the presence of EIgG, LPS-stimulated TNF-α mRNA shows a significant increase in stability. This increase in stability should increase the levels and availability of TNF-α mRNA for subsequent translation and secretion. A possible mechanism for increased TNF-α mRNA stability is a decrease in tristetraprolin (TTP) production (31). Macrophages deficient in this mRNA binding protein display augmented LPS-stimulated TNF-α secretion due to in-

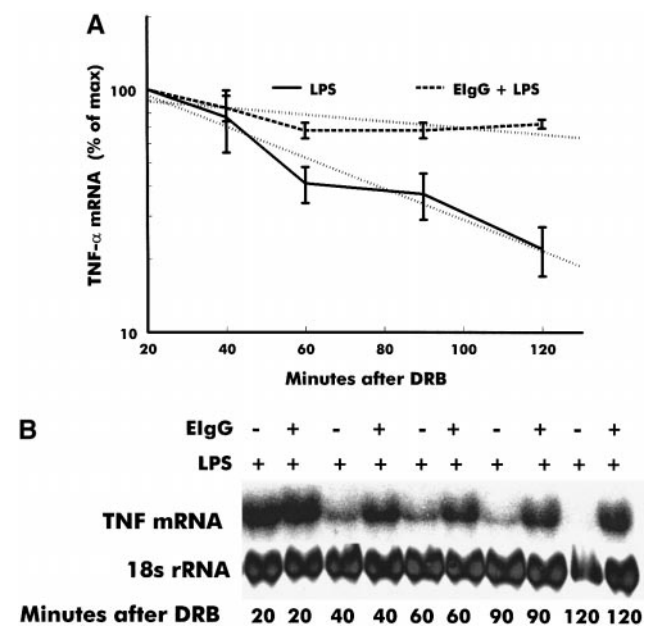

FIG. 5. Effect of EIgG on TNF-α mRNA stability. RAW 264.7 cells were incubated with LPS (100 ng/ml) or EIgG (3×10^6/ml) plus LPS. One hour after LPS, DRB (100 μM) was added and cells were lysed at the indicated times for RNA isolation and determination of mRNA levels by Northern blot analysis (A). A representative Northern blot is shown (B). Linear regression lines are shown for both conditions as dotted lines. Values are expressed as the percent of the TNF-α mRNA at 20 min after the addition of DRB (i.e., 80 min after LPS). Values are the mean $\pm$ the standard error of the mean for 3 experiments.

creased TNF-α mRNA stability. The effect of FcγR signaling on TTP production is presently unknown, but we are currently pursuing this question.

The role of TNF-α in the pathogenesis of several diseases makes it important to understand the factors that can augment the secretion of this cytokine. The present study indicates that immune complexes can prime macrophages for an exaggerated TNF-α response to LPS. In addition, we have recently shown that EIgG can also augment the TNF-α response to bacterial infection (32). Extrapolation of these findings suggest that autoimmune diseases and other pathologies that have circulating immune complexes may be at risk for enhanced TNF-α secretion in response to a bacterial infection (3). While immune complexes alone can stimulate TNF-α secretion by macrophages, the results of the present study suggest that immune complexes can prime phagocytes for an augmented TNF-α response to LPS or bacterial infection.

ACKNOWLEDGMENTS

The authors thank Luis Rodriguez for technical assistance and Wendy Hobb for editorial assistance. This study was supported by National Institutes of General Medical Sciences Grant GM-50368.

REFERENCES

1. Beutler, B. (1995) *J. Invest. Med.* **43,** 227–235.
2. Tracey, K. J., and Cerami, A. (1994) *Annu. Rev. Med.* **45,** 491–503.
3. Feldmann, M., Elliott, M. J., Woody, J. N., and Maini, R. N. (1997) *Adv. Immunol.* **64,** 283–350.
4. Watson, R. W., Redmond, H. P., and Bouchier-Hayes, D. (1994) *J. Leukocyte Biol.* **56,** 95–103.
5. Fenton, M. J., and Golenblock, D. T. (1998) *J. Leukocyte Biol.* **64,** 25–32.
6. Bundschuh, D. S., Barsig, J., Hartung, T., Randow, F., Docke, W.-D., Volk, H.-D., and Wendel, A. (1997) *J. Immunol.* **158,** 2862–2871.
7. Chapoval, A. I., Kamdar, S. J., Kremlev, S. G., and Evans, R. (1998) *J. Leukocyte Biol.* **63,** 245–252.
8. Donnelly, R. P., Freeman, S. L., and Hayes, M. P. (1995) *J. Immunol.* **155,** 1420–1427.
9. Hayes, M. P., Freeman, S. L., and Donnelly, R. P. (1995) *Cytokine* **7,** 427–435.
10. Richard, C. A. H., Gudewicz, P. W., and Loegering, D. J. (1999) *Am. J. Physiol.* **276,** R171–R177.
11. Kindt, G. C., Moore, S. A., She, Z. W., and Wewers, M. D. (1993) *Am. J. Physiol.* **265,** L178–L185.
12. Polat, G. L., Laufer, J., Fabian, I., and Passwell, J. H. (1993) *Immunology* **80,** 287–292.
13. Stein, M., and Gordon, S. (1991) *Eur. J. Immunol.* **21,** 431–437.
14. Commins, L. M., Loegering, D. J., and Gudewicz, P. W. (1990) *Inflammation* **14,** 705–715.
15. Lennartz, M. R., Lefkowith, J. B., Bromley, F. A., and Brown, E. J. (1993) *J. Leukocyte Biol.* **54,** 389–398.
16. Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1670–1674.
17. Sung, S. J., Walters, J. A., and Fu, S. M. (1992) *J. Exp. Med.* **176,** 897–901.
18. Loegering, D. J., and Blumenstock, F. A. (1985) *Infect. Immun.* **47,** 659–664.
19. Loegering, D. J., Commins, L. M., Minnear, F. L., Gary, L. A., and Hill, L. A. (1987) *Infect. Immun.* **55,** 2074–2080.
20. Loegering, D. J., Raley, M. J., Reho, T. A., and Eaton, J. W. (1996) *J. Leukocyte Biol.* **59,** 357–362.
21. Schwacha, M. G., Gudewicz, P. W., Snyder, J. A., and Loegering, D. J. (1993) *J. Immunol.* **150,** 236–245.
22. Berger, S., Ballo, H., and Stutte, H. J. (1996) *Eur. J. Immunol.* **26,** 1297–1301.
23. Foreback, J. L., Remick, D. G., Crockett-Torabi, E., and Ward, P. A. (1997) *Inflammation* **21,** 501–517.
24. Krutmann, J., Kirnbauer, R., Kock, A., Schwarz, T., Schopf, E., May, L. T., Sehgal, R. B., and Luger, T. A. (1990) *J. Immunol.* **145,** 1337–1342.
25. Selvan, R. S., Zhou, L., and Krangel, M. S. (1997) *Eur. J. Immunol.* **27,** 687–694.
26. Szabo, G., Kodys, K., and Miller-Graziano, C. L. (1991) *J. Clin. Immunol.* **11,** 326–335.
27. Tripp, C. S., Beckerman, K. P., and Uanue, E. R. (1995) *J. Clin. Invest.* **95,** 1628–1634.
28. Marsh, C. B., Pope, H. A., and Wewers, M. D. (1994) *J. Immunol.* **152,** 4604–4611.
29. Sutterwala, F. S., Noel, G. J., Salgame, P., and Mosser, D. M. (1998) *J. Exp. Med.* **188,** 217–222.
30. Sutterwala, F. S., Noel, G. J., Clynes, R., and Mosser, D. M. (1997) *J. Exp. Med.* **185,** 1977–1985.
31. Carballo, E., Lai, W. S., and Blackshear, P. J. (1998) *Science* **281,** 1001–1005.
32. Loegering, D. J., and Refici, M. L. (1999) *Shock* **11,** 23.