Amplitude and Frequency Modulation of Metabolic Signals in Leukocytes: Synergistic Role of IFN-γ in IL-6- and IL-2-Mediated Cell Activation[1]

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Many stimuli cause intracellular concentration oscillations of second messengers or metabolites, which, in turn, may encode information in their amplitudes and frequencies. We now test the hypothesis that synergistic cellular responses to dual cytokine exposure correlate with cross-talk between metabolic signaling pathways of leukocytes. Polarized RAW264.7 macrophages and human neutrophils and monocytes exhibited NAD(P)H autofluorescence oscillation periods of ≅20 s. IFN-γ tripled the NAD(P)H oscillatory amplitude for these cells. Although IL-6 had no effect, incubation of cells with IFN-γ and IL-6 increased both oscillatory amplitude and frequency. Parallel changes were noted after treatment with IFN-γ and IL-2. However, IL-1β and TNF-α did not display frequency doubling with or without IFN-γ exposure. To determine whether frequency doubling required complete IFN-γ signaling or simply metabolic amplitude modulation, an electric field was applied to cells at NAD(P)H troughs, which has been shown to enhance NAD(P)H amplitudes. Electric field application led to frequency doubling in the presence of IL-6 or IL-2 alone, suggesting that amplitude modulation is crucial to synergism. Because NADPH participates in electron trafficking to NO, we tested NO production during cytokine exposure. Although IL-6 and IL-2 alone had no effect, IFN-γ plus IL-6 and IFN-γ plus IL-2 enhanced NO release in comparison to IFN-γ treatment alone. When NO production was examined for single cells, it incrementally increased with the same phase and period as NAD(P)H. We suggest that amplitude and frequency modulation of cellular metabolic oscillations contribute to intracellular signaling synergy and entrain NO production. ***The Journal of Immunology*****, 1999, 163: 4367–4374.**

Information transduction across plasma membranes is often envisioned as the appearance of a second messenger such as cAMP, calcium, or phosphoproteins within the cytoplasm (1). In addition to a compound's presence, its concentration can vary temporally. For example, relative changes in intracellular calcium concentration or amplitude can lead to differential gene expression (2). Intracellular calcium concentrations oscillate as well, with information apparently frequency-encoded (3–7). Oscillations in pyridine nucleotides have been observed and may be linked to calcium oscillations and downstream cell functions (8–11). Thus, a rich nonlinear dynamic paradigm is emerging in the study of cellular signal transduction and processing in response to external stimuli.

We have proposed that amplitude and frequency modulation of intracellular metabolite (e.g., NADPH) concentrations may encode signaling information (11–13). We have speculated, for certain energy-demanding leukocyte functions (e.g., migration and adherence), that cell metabolite flux is the chemical signal for a cell function to appear while the associated kinases and phosphatases are the conduit through which the signal passes (11). For example, NADPH oxidase phosphorylation may "tune" the oxidase's K_d to intercept the "signal" metabolic oscillations, which are then decoded into a series of superoxide bursts (12). Superoxide oscillations may, in turn, explain the multiple, periodic cytolytic events observed during cell-mediated cytotoxicity (13). This interpretation is in agreement with the finding that NAD(P)H oscillations are in-phase with oxidative oscillations (12) and that superoxide production follows NAD(P)H amplitude and frequency changes (12, 14). Thus, neutrophil metabolic oscillations may be linked to cellular functions such as kinase/phosphatase pathways, oxidant production, pericellular proteolysis, cytolysis, and microfilament extension (11–13). Furthermore, neutrophils optimally detect time-varying chemical fields displaying this same period (15). Cells are also capable of detecting external electric fields when frequency and phase matched with intracellular metabolic oscillators (16). If these oscillators are a central element in cell function, it should be possible to identify patients with abnormal metabolic oscillations. Indeed, neutrophils from pediatric-onset pyoderma gangrenosum patients display aberrations in intracellular oscillators and motility or shape that can be ameliorated by pharmacologic or physical perturbations that restore sinusoidal oscillations (17, 18). Hence, leukocyte metabolic clocks may contribute to cell signaling and drive certain functions.

This study explores the mechanism of cytokine synergy in cell activation. Although cytokine cooperation is well-known (19, 20), the mechanisms responsible for processing multiple signals is not. We show that prior enhancement of oscillatory metabolic amplitudes participates in IL-6- and IL-2-mediated metabolic frequency doubling (i.e., activation) effect on cells. Furthermore, metabolic oscillatory changes parallel downstream physiologic changes in NO production.

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Materials and Methods

Materials

Murine and human cytokines were obtained from R&D Systems (Minneapolis, MN). FMLP, PxB,[3] LPS (*Escherichia coli* serotype 026:B6), and cycloheximide were obtained from Sigma (St. Louis, MO). Diaminofluorescein-2 diacetate (DAF-2 DA) was obtained from Daiichi Kagaku Yakuhin (Tokyo, Japan).

Cell culture

RAW264.7 macrophages were grown in RPMI 1640 containing 10% FCS and 1% PSA (penicillin G/streptomycin/amphotericin B) (Life Technologies, Grand Island, NY). For spectrophotometric assays, cells were grown in 24-well plates. For microscopy experiments, cells were grown for 24 h attached to glass coverslips.

Leukocyte isolation

Peripheral blood monocytes and neutrophils were obtained using two Ficoll-Hypaque solutions of different buoyant densities (Histopaque 1077 and 1119; Sigma) and centrifugation. Cells were washed twice by centrifugation and then resuspended in HBSS (Life Technologies). Trypan blue staining indicated that 95–99% of the cells were viable.

Spectrophotometric assay for NO

Macrophages (10^6/ml) were placed on culture plates and treated with recombinant murine IFN-γ at 10 U/ml and with 1000 U/ml IL-2 or 25 ng/ml of recombinant murine IL-6, IL-1β, or TNF-α for 24 h. Cell-free culture supernatants were collected for NO measurement. NO release was determined by assaying supernatants for nitrite content. Briefly, 40 μl of cell-free supernatant was reacted for 10 min at room temperature with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diaminedihydrochloride, 2.5% phosphoric acid) as described (21). Optical densities were measured at 540 nm. Nitrite content was quantitated by comparison with a standard curve generated using sodium nitrite (0–100 μM).

Electrode configuration

Electric fields were applied using two parallel platinum electrodes or Ag/AgCl electrodes (MF-2063; Bioanalytical Systems, West Lafayette, IN) as described for optical microscopy (18, 22, 23). A power supply (Grass Medical Instruments, Quincy, MA) was used to apply a pulsed square wave DC electric fields at 2×10^{-3} V/m. Electric field intensities were determined by measuring the current (23) using an electrometer (model 6517A; Keithley Instuments, Cleveland, OH). Pulse application was performed manually to coincide with the trough in NAD(P)H autofluorescence intensity for each cell under study.

Microscopic assay for NO production

Single-cell NO production experiments were performed using 2% gelatin matrices, similar to that previously described (12). Fluid-phase gelatin was mixed with 15 μM DAF-2 DA at 45°C then allowed to cool to 37°C, where it is a semisolid. DAF-2 DA has been previously shown to become fluorescent upon exposure to NO, but not other reactive species such as superoxide and hydrogen peroxide (24).

Microscopy

Cells were examined using an axiovert fluorescence microscope (Carl Zeiss, New York, NY) with mercury illumination and a quartz epifluorescence condenser interfaced to a Perceptics Biovision system (Knoxville, TN) (11). Quantitative experiments were performed using a 40× objective to provide depth of field and a 37°C stage. NAD(P)H autofluorescence was detected using 365DF20 and 405DF35 filters and a 405 long-pass dichroic mirror (11). Fluorescence levels were quantitated using a Hamamatsu (Bridgewater, NJ) photomultiplier tube held in a Products for Research (Danvers, MA) housing coupled to a microscope (11). Cells were illuminated individually. Background photon count rates were taken from a neighboring area that contained no cells. A Mac 9600 computer and MacLab system were used to record and analyze kinetic changes in fluorescence levels.

[3] Abbreviations used in this paper: PxB, polymyxin B; DAF-2 DA, diaminofluorescein-2 diacetate.

Results

The physiological interpretation of intracellular signals can depend upon the amplitudes or frequencies of such signals (2–17). This study tests the hypothesis that cytokines affect metabolic signaling and that cytokine synergy is accompanied by cross-talk between amplitude and frequency changes in metabolic signals.

IFN-γ enhances oscillatory NAD(P)H amplitudes

Cell illumination at ~360 nm leads to autofluorescence emission (>400 nm) that is largely accounted for by pyridine nucleotides (NADH + NADPH) (25, 26). This physical attribute of NAD(P)H has been variously exploited to monitor metabolic oscillations (e.g., 3, 9–12, 14, 16–18). We have employed this strategy as a nonperturbative method to study metabolic signaling in response to cytokine stimulation.

Previous studies have suggested that IFN-γ, IL-6, IL-2, IL-1β, and TNF-α can prime or activate cell functions, often in synergy with other ligands (e.g., 19, 20, 27–32). Enhanced leukocyte function is presumably mediated by chemical events downstream from receptor ligation, which includes metabolic responses. To test the effects of cytokines on metabolic oscillations, RAW264.7 macrophages were treated with various cytokines (Table I). In contrast to neutrophil-activating substances such as FMLP, immune complexes, and yeast (11, 14, 33), these murine cytokines did not influence NAD(P)H oscillation periods (Table I). Although IL-6, IL-2, IL-1β, and TNF-α did not affect NAD(P)H oscillations, cell exposure to IFN-γ did significantly increase the amplitude of metabolic oscillations ($p < 0.001$; line 8 vs line 3 in Table I). IFN-γ dose-response studies were conducted from 0–25 U/ml. A dose of 12.5 U/ml was found to be sufficient to induce a maximal change in NAD(P)H amplitude. However, as previously observed for other IFN-γ-mediated changes in cell physiology (19, 32, 34), this required an incubation time of 4 h at 37°C. Thus, IFN-γ affects the amplitude of metabolic oscillations in RAW264.7 cells.

Single-cell kinetic experiments were conducted to better define the time-frame required for IFN-γ-mediated NAD(P)H amplitude modulation. Cells were first exposed to IFN-γ for ~3.5 h in an incubator then transferred to a microscope stage at 37°C. As shown in Fig. 1*A* and Table I, IFN-γ dramatically increased the amplitude of NAD(P)H oscillations. These amplitude changes progressed quickly to a steady-state level. However, when cycloheximide (0.5 μg/ml) was added with IFN-γ, no changes in amplitude were observed (Table I). Thus, protein synthesis appears to be required for NAD(P)H amplitude modulation by IFN-γ, which is also consistent with the 4 h incubation time required for these changes.

IFN-γ primes macrophages for IL-6- and IL-2-mediated metabolic frequency doubling

Our previous studies (11–14, 33) suggest that metabolic frequency doubling is a hallmark of leukocyte activation. For example, FMLP, immune complexes, and β-glucans increase metabolic oscillation frequency, whereas certain antiinflammatory agents prevent these changes. Although frequency changes were not observed when cells were incubated with murine IL-6, IL-2, IL-1β, or TNF-α alone, we hypothesized that signaling events associated with dual cytokine exposure could synergize to yield outputs that neither cytokine alone possessed. This is consistent with numerous studies illustrating cytokine synergy (e.g., 19, 20). Because previous studies established that IFN-γ primes leukocytes (e.g., 31), we first exposed cells to IFN-γ (4 h at 15 U/ml) followed by the addition of a second cytokine. The addition of IL-6 to IFN-γ-treated macrophages led to frequency doubling within 3 min (Fig. 1*A*, Table I). Similarly, exposure of IFN-γ-primed cells to IL-2 (50

Table I. *NAD(P)H oscillations of macrophages in response to external stimuli*[a]

Exposure	Stimulus 1	Stimulus 2	*n*	*cn*	Amplitude (kcount/s)	Period (s)
LPS	1. LPS	None	5	100	104 ± 34	11.9 ± 1.8
	2. LPS + PxB	None	5	80	103 ± 25	22.9 ± 2.8
Single cytokine	3. None	None	5	225	100 ± 31	22.8 ± 2.5
	4. IL-6	None	4	80	104 ± 32	23.1 ± 1.9
	5. IL-2	None	3	80	102 ± 21	22.9 ± 2.0
	6. IL-1β	None	4	80	99 ± 28	22.6 ± 2.2
	7. TNF-α	None	4	120	99 ± 28	22.6 ± 2.3
	8. IFN-γ[1]	None	12	246	284 ± 38	23.0 ± 2.3
	9. IFN-γ + CHX	None	4	140	100 ± 23	22.9 ± 2.3
Dual cytokine	10. IFN-γ	IL-6	16	312	273 ± 42	11.9 ± 1.4
	11. IFN-γ	IL-2	3	90	269 ± 33	11.7 ± 1.4
	12. IL-6	IFN-γ	6	114	281 ± 34	21.9 ± 2.3
	13. IFN-γ	IL-1β	5	140	291 ± 41	22.6 ± 2.1
	14. IFN-γ	TNF-α	5	122	269 ± 39	23.0 ± 2.2
Electric field	15. Electric field	None	24	135	279 ± 42	22.8 ± 2.1
	16. Electric field	IL-6	21	89	275 ± 34	12.1 ± 1.9
	17. Electric field	IL-2	3	80	102 ± 21	22.9 ± 2.0
	18. Electric field + CHX	None	6	135	280 ± 28	22.7 ± 2.2
	19. Electric field + CHX	IL-6	4	80	279 ± 34	11.9 ± 1.8
	20. Electric field + CHX	IL-2	3	50	290 ± 29	11.8 ± 1.7
	21. Electric field	TNF-α	17	51	270 ± 37	22.6 ± 2.4
	22. Electric field	IL-1β	18	48	279 ± 41	23.0 ± 1.9
	23. IFN-γ	Electric field	22	65	275 ± 39	22.9 ± 2.0
	24. IL-6	Electric field	11	85	281 ± 35	11.8 ± 1.7

[a] The values of *n* and *cn* represent the number of independent experiments and the number of cells quantitated, respectively. For the experiments listed in line 2, LPS and PxB were mixed prior to incubations with cells. Incubations with IFN-γ were performed for 4 h at 37°C. On line 9, CHX and IFN-γ were added simultaneously to cells. CHX was added prior to cytokine exposure for the experiments listed on lines 19 and 20. Additional details and reagent concentrations are listed in the text.

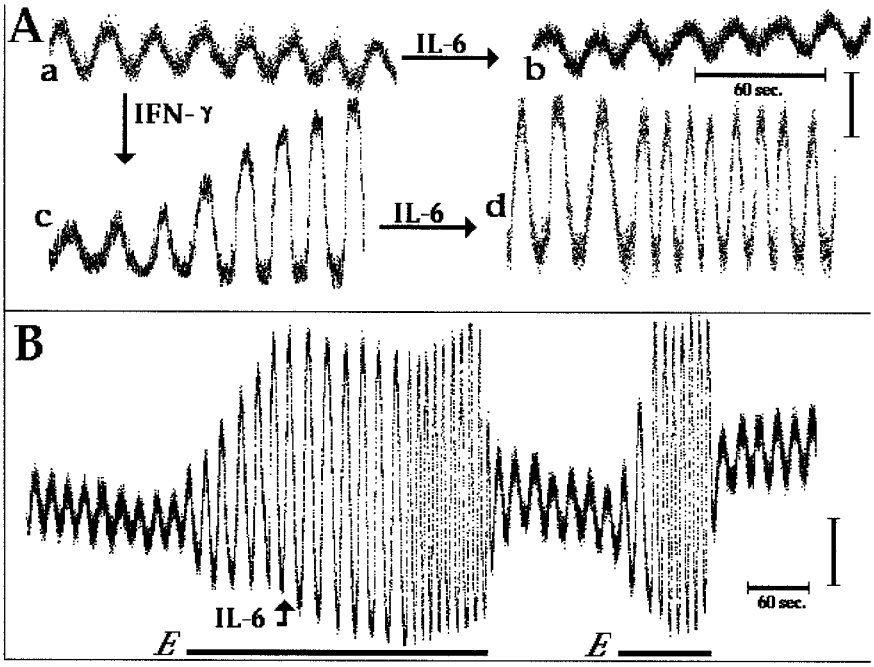

FIGURE 1. Representative kinetic traces illustrating the effects of IFN-γ and IL-6 exposure on metabolic oscillations of RAW264.7 macrophages. These traces show NAD(P)H autofluorescence intensity (ordinate) vs time (abscissa). Polarized cells were studied on glass slides at 37°C. *A,* Untreated cells demonstrated NAD(P)H oscillations with a period of about 20 s (*trace a*). The NAD(P)H oscillatory properties of cells were not effected by treatment with IL-6 (25 ng/ml) (*trace b*). However, the addition of IFN-γ (15 U/ml, 4 h) increased NAD(P)H amplitude (*trace c*). To illustrate the switch in amplitudes, cells were continuously monitored after an incubation period of 3 h 45 min. Only a small time interval corresponding to the amplitude change is shown. After exposure to IFN-γ (15 U/ml, 4 h), IL-6 (25 ng/ml) triggered metabolic frequency doubling (*trace d*). Again, this panel only shows the time interval corresponding to the frequency doubling event (about 3 min after IL-6 addition). *B,* The synergy of IL-6 and electric fields in metabolic oscillation frequency doubling. A representative trace covering a 7-min period is shown. A phase-matched electric field was applied at NAD(P)H troughs (long horizontal bars) resulting in enhanced NAD(P)H amplitudes. IL-6 was added at the indicated time point. About 3 min after IL-6 addition in the presence of an appropriate electric field, NAD(P)H oscillation frequency was increased 2-fold. (Correspondingly, the frequency of pulsed electric field application was increased 2-fold to match the NAD(P)H troughs.) Low-amplitude 20-s oscillations immediately returned when the field was terminated. The frequency doubling effect returned when the field was restored. In both panels, the vertical bars represent 50 kcounts/s, and the horizontal bars indicate 60 s.

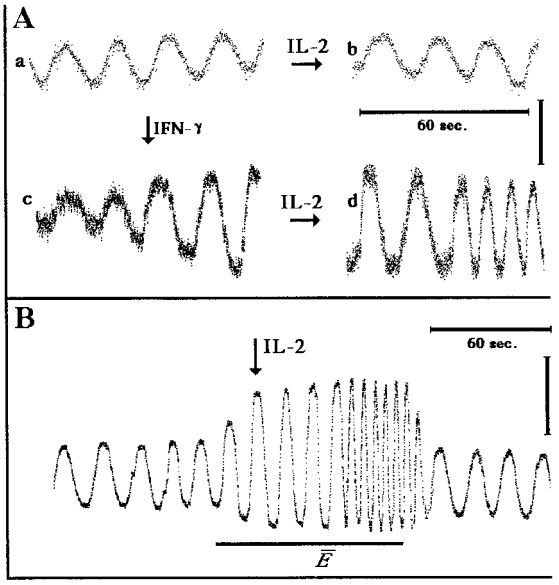

FIGURE 2. The effects of IFN-γ and IL-2 exposure on metabolic oscillations of RAW264.7 macrophages. *A,* Untreated cells demonstrated NAD(P)H oscillations with a period of about 20 s (*trace a*). The NAD(P)H oscillatory properties of cells were not effected by treatment with IL-2 (50 ng/ml) (*trace b*). However, the addition of IFN-γ (15 U/ml, 4 h) increased NAD(P)H amplitude, as also noted in Fig. 1 (*trace c*). After exposure to IFN-γ, IL-2 (50 ng/ml) triggered metabolic frequency doubling (*trace d*). This panel only shows the time interval corresponding to the frequency doubling event. *B,* The synergy of IL-2 and electric fields in metabolic oscillation frequency doubling. A representative trace covering a 6-min period is shown. A phase-matched electric field was applied at NAD(P)H troughs (long horizontal bars) resulting in enhanced NAD(P)H amplitudes. IL-2 was added at the indicated time point. About 1–2 min after IL-2 addition in the presence of an appropriate electric field, NAD(P)H oscillation frequency was increased 2-fold. Low-amplitude 20-s oscillations immediately returned when the field was terminated. In both panels, the vertical bars represent 50 kcounts/s, and the horizontal bars indicate 60 s.

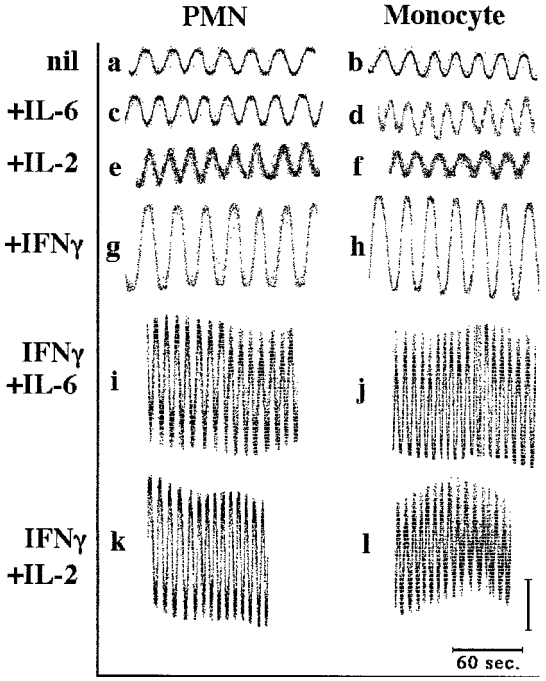

FIGURE 3. Synergistic effects of cytokines on metabolic signaling. Representative kinetic traces of NAD(P)H autofluorescence oscillations of neutrophils (*left*) and monocytes (*right*) are shown. Although IL-6 (25 ng/ml) and IL-2 (50 ng/ml for neutrophils and 100 ng/ml for monocytes) had no effect on NAD(P)H oscillations (*traces c–f*), IFN-γ tripled the amplitude of metabolic oscillations (*traces g* and *h*). However, when IL-6 or IL-2 was added to IFN-γ-primed cells, frequency doubling was observed (*traces i–l*). The vertical bar represents 50 kcounts/s, whereas the horizontal bar shows 60 s.

ng/ml) led to metabolic frequency doubling within 3 min (Fig. 2*A*, Table I). Specificity is suggested by the fact that IL-1β and TNF-α had no effect on IFN-γ-primed macrophages (Table I). Thus, IFN-γ and IL-6 or IFN-γ and IL-2 synergize to increase the amplitude and frequency of NAD(P)H oscillations in macrophages.

Previous studies have shown that IFN-γ and LPS can synergistically enhance NO production by macrophages (e.g., 20, 32). Therefore, we examined the effect of LPS on NAD(P)H oscillations. LPS (100 ng/ml) doubled the frequency of metabolic oscillations without effecting the oscillation's amplitude (Table I). This behavior is similar to that of FMLP and immune complexes (11, 12, 14, 33). Although treatment with IL-6 or IL-2 alone did not effect metabolic oscillations, we were concerned that subthreshold LPS contamination in the cytokine preparations may have synergistically interacted with the IFN-γ-treated macrophages to double the metabolic oscillation frequency. To deflect this concern, we included PxB (2 μg/ml) in these assays. PxB blocks the frequency doubling effect of LPS on cell metabolism (Table I). Moreover, PxB inclusion in the presence of IFN-γ and IL-6 or IFN-γ and IL-2 had no effect on metabolic amplitude or frequency (data not shown). Thus, LPS contamination cannot explain the frequency doubling effects of IL-6 and IL-2 on NAD(P)H oscillations.

IFN-γ primes human leukocytes for IL-6- and IL-2-mediated metabolic frequency doubling

The studies described above show that IFN-γ increases the amplitude of NAD(P)H oscillations in RAW264.7 macrophages and that IFN-γ can synergize with certain cytokines to double metabolic oscillation frequency. Although alterations in metabolic amplitude and frequency have been noted in human neutrophils (11, 12, 14, 16–18), it remains possible that these synergistic cytokine effects on metabolic signaling are limited to this transformed cell line. To test this possibility, we exposed human neutrophils and monocytes to human IFN-γ, IL-6, and/or IL-2. Human cytokines were employed in this system because murine IL-6 has no effect on human leukocytes (30). Fig. 3 shows a series of experiments illustrating the effects of cytokine exposure on metabolic oscillations of neutrophils and monocytes. Polarized neutrophils and monocytes show NAD(P)H autofluorescence oscillations of ~20 s that are not inflluenced by the addition of IL-6 (25 ng/ml) or IL-2 (50–100 ng/ml). However, exposure to IFN-γ (30 μg/ml) for >4 h led to increased metabolic oscillation amplitudes. Subsequent treatment of IFN-γ-primed cells with IL-6 or IL-2 led to high-amplitude, high-frequency metabolic oscillations. Thus, cytokine synergy is associated with metabolic cross-talk in multiple cell types.

Artificial metabolic amplitude modulation reconstitutes IL-6- and IL-2-mediated frequency doubling

Although the above studies correlate IFN-γ-mediated amplitude modulation with IL-6- and IL-2-mediated frequency doubling, it is unclear whether these changes require amplitude modulation or an independent parameter whose expression simply parallels that of NAD(P)H amplitude modulation. To address this issue, we sought to heighten NAD(P)H amplitudes by independent means. This was accomplished by exposing macrophages to a frequency and phase-matched electric field that heightens NAD(P)H oscillatory amplitudes in the absence of receptor ligation (12, 14, 16). Pulsed DC

Table II. *Macrophage production of NO*[a]

	NO (μM)	
	w/o PxB	PxB
1. Blank	0.83 ± 0	0.64 ± 0
2. LPS	26.8 ± 0.4	0.45 ± 0.3
3. IFN-γ	18.4 ± 0.67	11.3 ± 0.3
4. IL-1β	0.92 ± 0.13	0.73 ± 0.13
5. TNF-α	1.02 ± 0	2.45 ± 1.48
6. IL-6	7.49 ± 0.27	0.92 ± 0.4
7. IL-2	16.6 ± 0.65	1.33 ± 0.22
8. IFN-γ + IL-1β	18.4 ± 0.4	9.21 ± 0.27
9. IFN-γ + TNF-α	21.1 ± 0.4	12.3 ± 1.08
10. IFN-γ + IL-6	23.7 ± 0.27	16.6 ± 0.54
11. IFN-γ + IL-2	24.8 ± 0.87	15.1 ± 0.65

[a] The concentrations of cytokines used were: 10 U/ml IFN-γ, 25 ng/ml IL-1β, 25 ng/ml TNF-α, 25 ng/ml IL-6, and 1000 U/ml IL-2. LPS was used at a concentration of 100 ng/ml. The data shown are the mean ± SD of three independent experiments, each performed in triplicate. Values of p in the presence of PxB were as follows: line 1 vs line 3, $p < 0.01$; line 3 vs line 10, $p < 0.01$; line 3 vs line 11, $p < 0.01$. Comparisons between lines 3 and 8 and lines 3 and 9 were not significantly different.

electric fields (2×10^{-3} V/m with 20 ms duration) were applied to cells at troughs of NAD(P)H autofluorescence intensity (12, 14, 16). Data are shown in Fig. 1*B* and Table I. As Fig. 1*B* shows, the NAD(P)H oscillatory amplitude markedly increases in the presence of an appropriate phase-matched electric field; maximal peak height is reached within <2 min. About 2–3 min after IL-6 addition, the metabolic frequency doubles ($\tau \cong 10$ s). When the electric field is terminated, the high-amplitude, high-frequency oscillations rapidly revert to the low-amplitude, low-frequency state. Importantly, these parallel amplitude/frequency modulation events are reversible. As shown later in this same trace, when the electric field is again applied to this same cell, the frequency doubling reappears immediately. Similar findings were observed for all cells. Moreover, exposure of cycloheximide-pretreated cells to electric fields elicited the same changes in NAD(P)H amplitude and IL-6-dependent frequency alterations (Table I). Parallel experiments using IL-2 and electric field application yielded indistinguishable results (Fig. 2*B*, Table I). Electric field and IFN-γ-mediated NAD(P)H amplitude modulation differ in both their induction period and protein synthesis dependence. We suggest that metabolic amplitude modulation, in the absence of IFN-γ receptor ligation or altered gene expression, is sufficient to synergize with IL-6 or IL-2 to elicit metabolic frequency doubling.

IFN-γ and IL-6 or IL-2 synergize to increase macrophage NO production

To test the physiological role of amplitude and frequency changes, we measured NO production by RAW264.7 macrophages in response to various cytokine treatments. Experiments were conducted in the presence and absence of PxB to control for the potential effect of LPS contamination. LPS (100 ng/ml) induced NO production by macrophages, but was blocked by PxB (2 μg/ml) (Table II). The murine cytokines IL-6, IL-1β, and IL-2, when incubated with macrophages, had no significant effect on NO production as judged by nitrite formation (Table II). However, IFN-γ (10 U/ml) increased NO release to 11.3 ± 0.3 μM in the presence of PxB; this confirms the fact that IFN-γ is responsible for increased NO production. Cell exposure to IL-1β or TNF-α after treatment with IFN-γ gave results similar to that of IFN-γ alone when PxB was included during cell treatment (Table II). In contrast, IL-6 and IL-2 were found to synergize with IFN-γ to potentiate NO release (Table II), which could not be explained by LPS contamination. Thus, certain cytokine combinations can synergize to enhance NO release.

Single-cell measurements of NO release

The studies described above correlate: 1) IFN-γ-mediated increases in NAD(P)H amplitude with heightened NO production and 2) IL-6- and IL-2-mediated metabolic frequency doubling with enhanced NO production. To more closely link metabolic oscillations with NO production, we assessed NO production by single cells. Cells were observed on microscope slides at 37°C surrounded by a gelatin matrix containing DAF-2 DA, which reports local NO availability. The gel, which resembles an extracellular matrix, limits the extent of probe diffusion during experimentation. Microfluorometric observations of IFN-γ (15 U/ml, 4 h)- and IL-6 (25 ng/ml)-treated cells revealed a stepwise increase in NO-mediated accumulation of pericellular fluorescence (Fig. 4*A*, *trace c*). However, treatment of cells with buffer alone caused little or no NO production (Fig. 4*A*, *trace a*; Fig. 4*B*, *trace a*). Exposure to IFN-γ alone (Fig. 4*A*, *trace b*; Fig. 4*B*, *trace b*) led to significant NO production, although at a lower frequency than that of IFN-γ- and IL-6-treated cells. Similarly, NO release doubling was also observed for cells treated with IFN-γ (15 U/ml, 4 h) and IL-2 (50 ng/ml) (Fig. 4*B*, *trace c*). Thus, the combination of IFN-γ with either IL-6 or IL-2 leads to heightened NO release. Moreover, the frequency of NO release matches that previously reported for neutrophil NAD(P)H and superoxide production oscillations (12).

We next tested the ability of cytokines to influence NO release from human leukocytes using these cytokines. Human monocytes are capable of producing NO (35). Although treatment with buffer alone had no effect on monocytes (Fig. 4*C*, *trace a*), IFN-γ (15 U/ml, 4 h) did have a small effect on local NO release (Fig. 4*C*, *trace b*). As noted above for RAW264.7 cells, exposure to IFN-γ (15 U/ml, 4 h) and IL-6 (25 ng/ml) led to increased NO release (Fig. 4*C*, *trace c*). However, due to the fact that normal leukocytes produce smaller quantities of NO than RAW264.7 cells, the traces have a lower intensity and higher noise level.

Relative phases of NAD(P)H oscillations and NO release revealed by single-cell measurements

To further link NAD(P)H oscillations with NO release, we simultaneously followed NAD(P)H autofluorescence and NO release using the techniques described above. These measurements were made by rapidly switching the interference filters between the setups for NAD(P)H autofluorescence and NO fluorescence emission detection, resulting in the staggered appearance of the data (Fig. 5). Data are shown for IFN-γ (15 U/ml, 4 h)-treated (Fig. 5*A*) and IFN-γ (15 U/ml, 4 h) plus IL-6 (25 ng/ml)-treated (Fig. 5*B*) RAW264.7 cells. Parallel observations of NAD(P)H and NO production oscillations from the same cells were recorded. Both NAD(P)H autofluorescence and NO release were observed at the same frequency for cells under both conditions. Moreover, the increases in DAF-2 DA fluorescence were observed near NAD(P)H oscillation peaks for both IFN-γ (15 U/ml, 4 h) (Fig. 5*A*), IFN-γ plus IL-6 (Fig. 5*B*), and IFN-γ plus IL-2 (data not shown) treatments. Thus, the phase of NO production increments appear to parallel the points of maximal NAD(P)H concentrations.

Discussion

Although the molecular events participating in transmembrane signaling are becoming well-known (1), the mechanisms cells use to handle or process this information to make decisions are not generally understood. We have emphasized the importance of intracellular oscillators and their temporal properties (period, amplitude, and phase) in normal leukocyte function and disease (11–18).

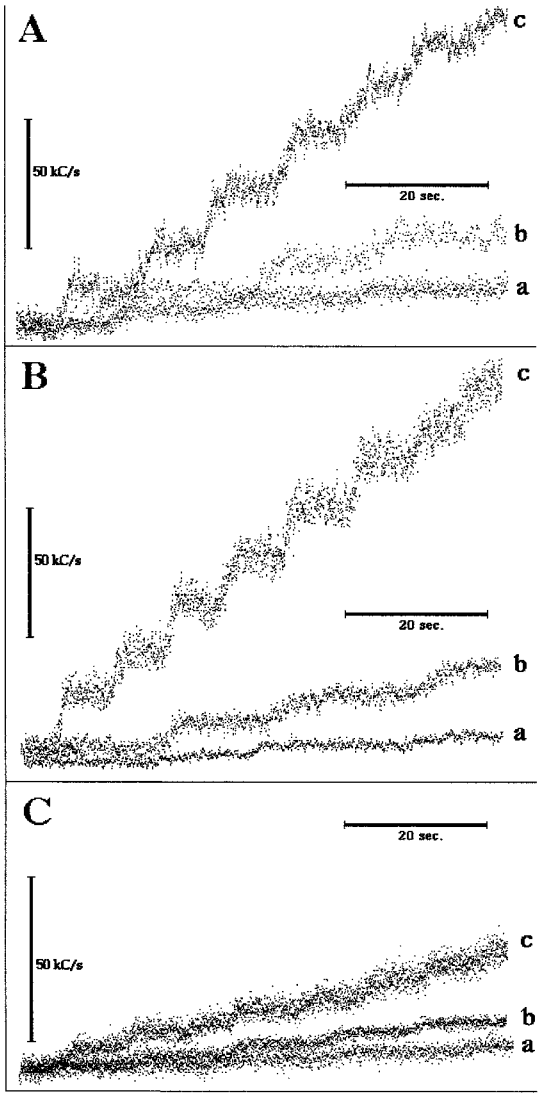

FIGURE 4. Representative kinetic traces showing the properties of NO production from individual RAW264.7 macrophages. These traces show fluorescence intensity at the ordinate vs time (abscissa). Polarized cells were examined at 37°C in gel matrices doped with 15 μM DAF-2 DA. Because DAF-2 DA becomes fluorescent upon exposure to NO, the total amount of fluorescence near individual cells increases with time. *A*, Cells treated with buffer alone (control, *trace a*) produce negligable amounts of NO. Cells exposed to IFN-γ (4 h) produce NO (*trace b*). However, at the single-cell level, NO production is nonlinear; pulses of NO release are observed at an interval of $\cong$20 s. After treatment with IFN-γ (4 h) and IL-6, macrophages produce greater amounts of NO at a higher frequency (t $\sim$10 s.) (*trace c*). This higher frequency may account for the enhancement of NO production (Table II). *B*, Macrophages exposed to only buffer alone (*trace a*) do not produce NO. Cells exposed to IFN-γ (4 h) produce significant levels of NO (*trace b*), as noted in *A*. After treatment with IFN-γ (4 h) and IL-2, cells produce greater amounts of NO at a higher frequency (t $\sim$10 s.) (*trace c*). *C*, The kinetics of NO production by human monocytes. Although little or no NO is produced by control or IFN-γ-treated monocytes (*traces a* and *b*), cells treated with IFN-γ and IL-6 produce significant levels of NO (*trace c*). Both the amplitude and signal-to-noise ratio are reduced in these experiments because monocytes produce less NO than the RAW264.7 macrophage cell line (vertical bars, 50 kcount/s; the gain is higher in *C*; horizontal bars, 20 s.).

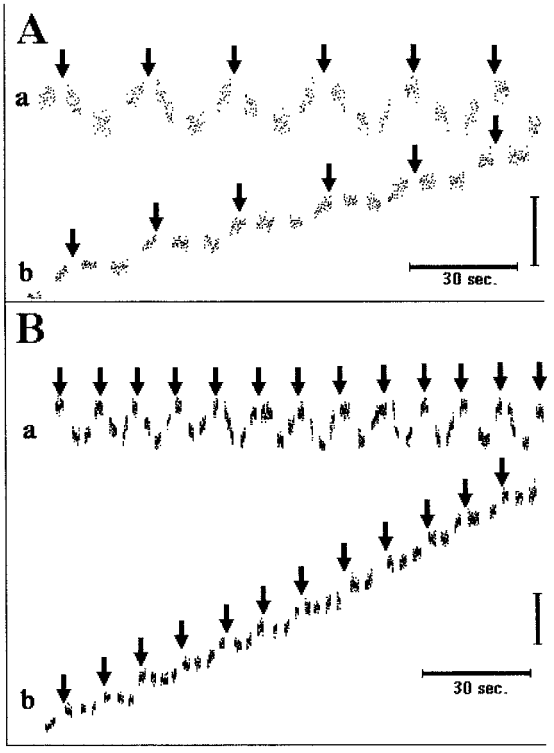

FIGURE 5. Relative phases of NAD(P)H oscillations and NO release for macrophages. *A*, Cells (IFN-γ for 4 h) were observed in gel matrices. The NAD(P)H autofluorescence (*trace a*) and the production of fluorescence from DAF-2 DA due to NO exposure (*trace b*) were measured for each cell. The optical set-ups for detection of NAD(P)H and NO production were alternated, thus creating the staggered photomultiplier tube output. By comparing *traces a* and *b*, the peak in NO release occurs at roughly the same time as the NAD(P)H peak, indicating that these two oscillators are approximately in phase. *B*, Cells were treated with IFN-γ for 4 h followed by exposure to IL-6 (25 ng/ml). The NAD(P)H autofluorescence frequency doubled to about 10 s (*trace a*), as noted above. In parallel, the frequency of NO release also doubled (*trace b*). Again, the peaks of NAD(P)H intensity correspond to NO production pulses (vertical bars, 50 kcount/s; horizontal bars, 30 s).

For example, the frequency of extracellular ligand concentration changes affects neutrophil turning behavior ("cell memory"), and the amplitude, frequency, and phase properties of applied electric fields affect NAD(P)H amplitudes and downstream oxidant production and DNA damage (14, 16). This study focused on the mechanism of cytokine cross-talk in leukocytes. Our results indicate that sequential amplitude and frequency modulation events accompany the IFN-γ/IL-6 and IFN-γ/IL-2 synergisms leading to enhanced NO production. Combined metabolic amplitude and frequency modulation represents a novel ligand information processing mechanism and may explain emergent physiological outcomes.

Recently, IFN-γ's mechanism of gene activation, via receptor-associated Janus kinase-mediated tyrosine phosphorylation of STAT proteins and STAT protein translocation to the nucleus, has been described (36, 37). However, the mechanisms linking the IFN-γ-responsive genes to phenotypic changes in cell behavior are uncertain. This signaling pathway leads to leukocyte priming for oxidant production and increased phagocytosis and tumoricidal activity (31, 38, 39). Inasmuch as a protein synthesis-dependent 4-h incubation period with IFN-γ is required for metabolic amplitude modulation, changes in gene expression are likely required for this process. We predict that metabolic pathways are affected by IFN-γ-mediated changes in gene expression. Metabolic amplitude modulation may account for certain phenotypic properties of IFN-γ-treated cells. We have previously shown that enhanced NAD(P)H amplitudes parallel heightened neutrophil spreading, extension,

and superoxide production (12, 14, 16). Thus, when the NADPH oxidase is "tuned" to the NADPH oscillatory region, the enhanced NADPH levels lead to greater oxidant production. NADPH amplitude modulation may be the physico-chemical mechanism responsible for the priming effects of IFN-γ (e.g., 31, 39). Greater levels of oxidant production may, in turn, explain the enhanced tumoricidal activity of neutrophils and macrophages. Inasmuch as ATP oscillates at the same frequency, but 180° out-of-phase with NAD(P)H (26), a similar argument may be relevant to IFN-γ-enhanced ATP-driven functions such as phagocytosis. Thus, metabolic amplitude modulation may provide the biochemical mechanism responsible for certain priming and effector functions of IFN-γ.

IL-6- and IL-2-mediated frequency doubling was dependent upon IFN-γ dose, IFN-γ incubation time, and protein synthesis. Although IFN-γ-mediated metabolic amplitude modulation accompanies IL-6- and IL-2-mediated frequency doubling, these experiments alone cannot establish amplitude modulation as a sufficient condition. We have found that application of electric fields to cells at troughs of NAD(P)H autofluorescence leads to enhanced NAD(P)H oscillatory amplitudes of untreated cells (14, 16). Therefore, we employed this strategy to alter NAD(P)H amplitudes in the absence of IFN-γ, receptor ligation, or its inducible gene products. We have shown that an applied electric field is able to substitute for IFN-γ treatment. That is, heightened NAD(P)H amplitudes are sufficient to reconstitute IL-6- and IL-2-mediated frequency doubling. These changes were observed regardless of the order of IL-6 or IL-2 addition with respect to electric field application and were reversible by switching the electric field on and off (Figs. 1*B* and 2*B*). We speculate that metabolic amplitude modulation is a key step in IFN-γ-mediated priming for IL-6- and IL-2-mediated metabolic signaling.

The mechanism(s) used by the IL-6 and IL-2 receptors to detect prior IFN-γ amplitude modulation is not known. However, a mechanism similar to that we have proposed for integrin/NADPH oxidase activation may be operating (11, 14). Briefly, the concentration of a substrate (e.g., NADPH or ATP) draws near the K_d of an important regulatory component, such as a receptor-associated kinase, thus allowing receptor function (such as metabolic signaling and NO production (Fig. 4)) to appear.

A physical characteristic of IL-6- and IL-2-mediated cell activation is metabolic frequency doubling. Similar responses have been observed for activating stimuli such as FMLP, immune complexes, β-glucans, and yeast (11, 14, 33). One physiological response accompanying NAD(P)H frequency doubling in response to dual cytokine exposure is a heightened NO production (Table II). Synergistic cytokine-mediated NO production enhancement was observed with IL-6 and IL-2; cytokines that did not trigger frequency doubling had no effect. Although this correlation is rigorous, it remains possible that all cells experience metabolic frequency doubling while only a small fraction of the cells experience heightened NO production. To deflect this concern, we evaluated NO production at the single-cell level. We found an incremental increase in NO production by individual cells that increased in parallel with NAD(P)H frequency (Fig. 4).

As noted above, Cox et al. (20) have observed a synergistic effect between IFN-γ and IL-2 that leads to a doubling of reactive nitrogen intermediate production and a dramatic increase in cell-mediated cytotoxicity. Although TNF-α may play a role in enhanced leukocyte function (20), TNF-α alone and TNF-α plus IFN-γ did not effect metabolic signaling in leukocytes. This suggests that TNF-α acts downstream from metabolic signaling.

We suggest that the increases in metabolic frequency and NO production increments are mechanistically related. The formation of NO and citrulline is catalyzed by NO synthase in the presence of NAD(P)H and molecular oxygen (40, 41). Because our buffers contained dissolved oxygen, we speculate that electron flux from NAD(P)H is rate limiting; thus, increasing the substrate oscillation frequency increases the NO production rate yielding the enhancement (IFN-γ vs IFN-γ plus IL-6 or IFN-γ plus IL-2). Importantly, the approximate phase alignment of NAD(P)H and NO production is consistent with an oscillatory metabolic apparatus entraining NO release (11, 12, 14, 16).

A potential implication of these studies concerns the mechanism of electric field-to-cell interactions. Our studies indicate that application of a low-frequency pulsed DC electric field mimics the NAD(P)H amplitude modulation effects obtained for IFN-γ-primed cells. Similarly, both exposure to an appropriate phase-matched electric field and IFN-γ lead to enhanced assembly of microfilaments (16, 42). The synergism of electric fields and IL-6 may cause reactive nitrogen and oxygen species production and DNA damage in vivo, which may be relevant to biological effects of electromagnetic fields.

Recently, there has been a great deal of interest in the potential role of biological oscillators as signaling devices (2–17). This study extends the field to include cytokine-mediated signaling by providing a potential mechanisms for IFN-γ-mediated phenotypic changes and IFN-γ/IL-6 and IFN-γ/IL-2 signaling synergy in leukocytes. This approach may provide a means of simplifying the bewildering array of cytokine interactions and dissecting their mechanisms of action.

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References

1. Petty, H. R. 1993. *Molecular Biology of Membranes.* Plenum Press, New York.
2. Dolmetsch, R. E., R. S. Lewis, C. C. Goodnow, and J. I. Healy. 1997. Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature 386:855.*
3. Goldbeter, A. 1996. *Biochemical Oscillations and Cellular Rhythms.* Cambridge University Press, Cambridge, U.K.
4. Tsien, R. W., and R. Y. Tsien. 1990. Calcium channels, stores, and oscillations. *Annu. Rev. Cell Biol. 6:715.*
5. Berridge, M. J. 1997. The AM and FM of calcium signalling. *Nature 386:759.*
6. De Koninck, P., and H. Schulman. 1998. Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. *Science 279:227.*
7. Gu, X., and N. C. Spitzer. 1995. Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. *Nature 375:784.*
8. Corkey, B. E., K. Tornheim, J. T. Deeney, M. C. Glennon, J. C. Parker, F. M. Matschinsky, N. B. Ruderman, and M. Prentki. 1988. Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. *J. Biol. Chem. 263:4254.*
9. Pralong, W.-F., C. Bartley, and C. B. Wollheim. 1990. Single islet β-cell stimulation by nutrients: relationship between pyridine nucleotides, cytosolic Ca^{2+} and secretion. *EMBO J. 9 53.*
10. O'Rourke, B., B. M. Ramza, and E. Marban. 1994. Oscillations of membrane current and excitability driven by metabolic oscillations in heart cells. *Science 265:962.*
11. Kindzelskii, A. L., M. M. Eszes, R. F. Todd III, and H. R. Petty 1997. Proximity oscillations of complement receptor type 4 and urokinase receptors on migrating neutrophils. *Biophys. J. 73:1777.*
12. Kindzelskii, A. L., M. J. Zhou, R. P. Haugland, L. A. Boxer, and H. R. Petty 1998. Oscillatory deposition of reactive oxygen metabolites and pericellular proteolysis during neutrophil migration through model extracellular matrices. *Biophys. J. 74:90.*
13. Kindzelskii, A. L., and H. R. Petty. 1999. Early membrane rupture events during neutrophil-mediated antibody-dependent tumor cell cytolysis. *J. Immunol. 162: 3188.*
14. Petty, H. R. 1999. Oscillatory signals in migrating neutrophils: effects of time-varying chemical and electrical fields. In *Self-Organized Biological Dynamics and Nonlinear Control by External Stimuli,* J. Walleczek, ed. Cambridge University Press, Cambridge, U.K.
15. Albrecht, E., and H. R. Petty. 1998. Cellular memory: neutrophil orientation reverses during temporally decreasing chemoattractant concentrations. *Proc. Natl. Acad. Sci., USA 95:5039.*
16. Petty, H. R., and A. L. Kindzelskii. 1997. Extremely low frequency electric fields promote metabolic resonance and cell extension during neutrophil migration. *J. Allergy Clin. Immunol. 99:S317.*

17. Adachi, Y., A. L. Kindzelskii, S. Shaya, G. Cookingham, E. C. Moore, R. F. Todd, and H. R. Petty. 1998. Aberrant neutrophil trafficking and metabolic oscillations in severe pyoderma gangrenosum. *J. Invest. Dermatol. 111:259.*
18. Shaya, S., A. L. Kindzelskii, J. Minor, E. C. Moore, R. F. Todd, and H. R. Petty. 1998. Aberrant integrin (CR4, $\alpha_x\beta_2$; CD11c/CD18) oscillations on neutrophils in a mild form of pyoderma gangrenosum. *J. Invest. Dermatol. 111:101.*
19. Chen, L., D. Novick, M. Rubinstein, and M. Revel. 1988. Recombinant interferon-β1 (interleukin-6) induces myeloid differentiation. *FEBS Lett. 239:299.*
20. Cox, G. W., G. Melillo, U. Chattopadhyay, D. Mullet, R. H. Fertel, and L. Varesio. 1992. Tumor necrosis factor-α-dependent production of reactive nitrogen intermediates mediates IFN-γ plus IL-2-induced murine macrophage tumoricidal activity. *J. Immunol. 149:3290.*
21. Steuhr, D. J., and C. Nathan. 1989. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med. 169:1543.*
22. Friend, A. W., E. D. Finch, and H. P. Schwan. 1975. Low frequency electric field induced changes in the shape and motility of amoebas. *Science 187:357.*
23. Poo, M.-M., W. J. H. Poo, and J. W. Lam. 1978. Lateral electrophoresis and diffusion of concanavalin A receptors in the membrane of embryonic muscle cell. *J. Cell Biol. 76:483.*
24. Kojima, H., K. Sakurai, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, and T. Nagano. 1998. Development of a fluorescent indicator for nitric oxide based on the fluorescein chromophore. *Chem. Pharm. Bull. 46:373.*
25. Chance, B., E. K. Pye, A. K. Ghosh, and B. Hess. 1973. *Biological and Biochemical Oscillators.* Academic Press, New York.
26. Hess, B., and A. Boiteux. 1971. Oscillatory phenomena in biochemistry. *Annu. Rev. Biochem. 40:237.*
27. Kharazmi, A., H. Nielsen, C. Rechnitzer, and K. Bendtzen. 1989. Interleukin 6 primes human neutrophil and monocyte oxidative burst response. *Immunol. Lett. 21:177.*
28. Borish, L., R. Rosenbaum, L. Albury, and S. Clark. 1989. Activation of neutrophils by recombinant interleukin 6. *Cell. Immunol. 121:280.*
29. Mullet, D., R. H. Fertel, D. Kniss, and G. W. Cox. 1997. An increase in intracellular cyclic AMP modulates nitric oxide production in IFN-γ-treated macrophages. *J. Immunol. 158:897.*
30. Callard, R., and A. Gearing. 1994. *The Cytokine Factsbook*. Academic Press, New York.
31. Berton, G., L. Zeni, M. A. Cassatella, and F. Rossi. 1986. Gamma interferon is able to enhance the oxidative metabolism of human neutrophils. *Biochem. Biophys. Res. Commun. 138: 1276.*
32. Ding, A. H., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *J. Immunol. 141:2407.*
33. Alit, A., A. K. Kindzelskii, J. Zanoni, J. N. Jarvis, and H. R. Petty (1999) C3 deposition on immune complexes diminishes the frequencies of metabolic, proteolytic and superoxide oscillations of migrating neutrophils. *Cell. Immunol. 194:47.*
34. Lehtonen, A., S. Matikainen, and I. Julkunen. 1997. Interferons up-regulate STAT1, STAT2, and IRF family transcription factor gene expression in human peripheral blood mononuclear cells and macrophages. *J. Immunol. 159:794.*
35. Aubry, J.-P., N. Dugas, S. Lecoanet-Henchoz, F. Quaaz, H. Zhao, J.-F. Delfraissy, P. Graber, J.-P. Kolb, B. Dugas, and J.-Y. Bonnefoy. 1997. The 25 kDa soluble CD23 activites type III constitutive nitric oxide synthase activity via CD11b and CD11c expressed by human monocytes. *J. Immunol. 159:614.*
36. Schindler, C., and J. E. Darnell. 1995. Transcriptional responses to polypeptide ligands: The JAK-STAT pathway. *Annu. Rev. Biochem. 64:621.*
37. Ihle, J. 1996. STATs: signal transducers and activators of transcription. *Cell 84:331.*
38. Pestka, S., J. A. Langer, K. C. Zoon, and C. E. Samuel. 1987. Interferons and their actions. *Annu. Rev. Biochem. 56:727.*
39. Lengyel, P. 1982. Biochemistry of interferons and their actions. *Annu. Rev. Biochem. 51:251.*
40. Perry, J. M., and M. A. Marletta. 1998. Effects of transition metals on nitric oxide synthase catalysis. *Proc. Natl. Acad. Sci. USA 95:11101.*
41. Witteveen, C. F. B., J. Giovanelli, M. B. Yim, R. Gachhui, D. J. Stuehr, and S. Kaufman. 1998. Reactivity of the flavin semiquinone of nitric oxide synthase in the oxygenation of arginine to N^G-hydroxyarginine, the first step of nitric oxide synthesis. *Biochem. Biophys. Res. Commun. 250:36.*
42. Pfeffer, L. M., E. Wang, and I. Tamm. 1980. Interferon effects on microfilament organization, cellular fibronectin distribution, and cell motility in human fibroblasts. *J. Cell Biol. 85:9.*