IFN-γ-Independent Autocrine Cytokine Regulatory Mechanism in Reprogramming of Macrophage Responses to Bacterial Lipopolysaccharide

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Macrophages are now well recognized to have a critical role in both innate and acquired immunity. The sentinel macrophage function is highly regulated and serves to allow for intrinsic plasticity of the innate immune responses to potential environmental signals. However, the mechanisms underlying the dynamic properties of the cellular arm of innate immunity are poorly understood. Therefore, we have conducted a series of in vitro studies to evaluate the contribution of immunoregulatory cytokines, such as IFN-γ, IL-10, and IL-12, in modulation of macrophage responses. We found that macrophages from IFN-γ knockout (IFN-γ−/−) mice exhibit only marginal LPS-induced TNF-α, IL-12, and NO responses, all of which can be fully restored in the presence of rIFN-γ. Pretreatment with substimulatory LPS concentrations led to reprogramming of IFN-γ−/− macrophage responses in a dose-dependent manner that manifested by an increased TNF-α and IL-12, but not NO, production upon the subsequent LPS challenge. These reprogramming effects were substantially attenuated and profoundly enhanced in macrophages from IL-12−/− and IL-10−/− mice, respectively, as compared with those modulated in macrophages from the congenic wild-type mice. LPS-dependent reprogramming was also fully reproduced in macrophages isolated from SCID mice after immunodepletion of NK cells.

Our data strongly imply that cytokine (TNF-α and IL-12), but not NO, responses in macrophages may, at least in part, be governed by an autocrine IFN-γ-independent regulatory mechanism reciprocally controlled by IL-10 and IL-12. This mechanism may serve as an alternative/coherent pathway to the canonical IFN-γ-dependent induction of antimicrobial and tumoricidal activity in macrophages. The Journal of Immunology, 2001, 167: 392–398.

In recent years, the pivotal role of tissue macrophages and NK cells of innate immunity in the initiation of adaptive or T cell-dependent immune responses has emerged (reviewed in Ref. 1). Differential modulation of immune responses is, in large part, controlled by the ability of macrophages to generate an array of cytokine responses, e.g., TNF-α, IL-10, IL-12, IL-18, and IFN-γ, early on after encountering a potential invading infectious agent. The interactive/regulatory responses mediated by these macrophage-derived cytokines are manifested by the formation of autocrine/paracrine cytokine regulatory networks that serve to control functional cooperation among different cellular components of the innate immunity. The importance of these regulatory mechanisms underly activation of the innate immune responses and generation of instructive communication signals that orchestrate and/or direct the acquired immunity is dictated by the requirement for various types of effector mechanisms to achieve the protective immunity from different microbial pathogens.

Since its discovery more than 30 years ago in 1965 (2), it has been unequivocally established that IFN-γ, also earlier termed as type II IFN or macrophage-activating factor, has the capacity to control activation of bactericidal and tumoricidal immunologic programs of monocytes/macrophages (3). IFN-γ regulates inflammatory and antimicrobial/tumoricidal potential of macrophages by up-regulation of MHC class I and class II protein expression, enhanced production of macrophage-derived mediators (TNF-α, IL-1, IL-12, NO), and down-regulation of the synthesis of anti-inflammatory mediators such as IL-10 (4–8). Therefore, IFN-γ can be truly considered as a pleiotropic cytokine that is involved in regulation of virtually all immune responses including host defense, inflammation, and autoimmunity (reviewed in Ref. 9).

In a more specific sense, IFN-γ plays an important integrating role in coordination of immune responses to a plethora of microenvironment signals. In this respect, the bias of both innate and acquired immunity can be controlled by IFN-γ-dependent pathways of cytokine networking that ultimately dictate the development of an appropriate immune response against a given microbial pathogen (10–12). Specifically, IFN-γ is clearly documented to play a pivotal role in modulation of the protective immunity against Mycobacterium tuberculosis, Leishmania major, Listeria monocytogenes, and Cryptococcus neoformans (13–16). Of importance, the early production of IFN-γ by immunocompetent cells can differentially be controlled by such macrophage-derived cytokines as TNF-α, IL-10, IL-12, and IL-18, suggesting the complexity of interactive regulatory pathways as dictating cytokine networks of the immune system (17–20).

Our recent data strongly support the concept of functional plasticity of the innate immune responses in macrophages that play an important role in conducting immunologic surveillance by tissue macrophages (21). We have described a reproducible phenomenon of LPS-dependent “reprogramming” of macrophages for altered
phenotypic responses to challenge with LPS and/or other bacterial constituents as assessed by TNF-α, IL-10, IL-12, and NO production. The plasticity displayed in the repertoire of macrophage-derived cytokines may play a critical role in regulation of innate immunity and subsequent modulation of Ag-specific immune responses to infection (21). Recent findings that macrophages themselves may represent an essential source of IFN-γ (20) prompted our current research to further elucidate the potential contribution of IFN-γ-dependent regulatory mechanisms and pathways to LPS-dependent reprogramming of cytokine and NO responses in macrophages. Our data describe a novel IFN-γ-independent autocrine regulatory mechanism of LPS-dependent reprogramming of macrophage cytokine (TNF-α and IL-12) potential that is partially controlled by IL-10 and IL-12 immunoregulatory cytokines.

Materials and Methods

Mice

Inbred C57BL/6 mice, C57BL/6 IFN-γ-deficient (IFN-γ−/−) mice, C57BL/6 IL-10-deficient (IL-10−/−) mice, and C57BL/6 IL-12-deficient (IL-12−/−) mice were all purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were kept in pathogen-free conditions and housed in microisolator cages with food and water provided ad libitum. Wild-type inbred BALB/c mice and T and B cell-deficient BALB/c SCID/scid mice were purchased from Charles River Laboratories (Wilmington, MA). SCID mice were housed in laminar airflow hoods and had a free access to autoclaved food and water. All animals were maintained with a 12-h light-dark cycle in the Laboratory Animal Care, Kansas University Medical Center certified by the American Association for Accreditation of Laboratory Care.

Reagents

Purified LPS from Escherichia coli O111:B4 was purchased from List Biologic Laboratories (Campbell, CA). Recombinant mouse IFN-γ (sp. act. 1 × 10^9 U/mg and endotoxin level <0.1 ng/mg of protein) was a gift from S. W. Russell (University of Kansas Medical Center, Kansas City, KS). The specific peptide inhibitor of IL-1β-converting enzyme (ICE, caspase-1), Ac-Tyr-Val-Ala-Asp-aldehyde, was purchased from Bachem Bioscience (Bubendorf, Switzerland). Recombinant mouse IL-18 Abs were obtained from PeproTech (Rocky Hill, NJ). Rat anti-mouse F4/80 mAb (Harlan Bioproducts for Science) reactive with mouse F4/80 antigen was purchased from Wako Chemicals (Richmond, VA). Protein A-purified rabbit anti-mouse IL-18 Abs were obtained from Pharmingen (Rocky Hill, NJ). Rat anti-mouse F4/80 mAb was purchased from Harlan Bioproducts for Science (Indianapolis, IN). Rat anti-Pan NK cell DX5 mAb was obtained from BD Pharmingen (San Diego, CA). Polyinosinic-polycytidylic acid; N6-monomethyl-L-arginine (L-NMMA) and ibuprofen were purchased from Sigma (St. Louis, MO).

Isolation and stimulation of macrophages

Mice of 8–12 wk old were used in all studies. Mice were i.v. injected with 1.5 ml of sterile 4% thioglycollate broth (Difco Laboratories, Detroit, MI), and the elicited peritoneal cells were harvested by peritoneal lavage 4 days later. For all experiments in this study, peritoneal macrophages were further purified to limit the potential paracrine effects of other immunocompetent cells on LPS-dependent macrophage responses in vitro. For this purpose, we used a custom-designed murine macrophage Ab cocktail against B220, CD2, TER119, Gr-1, and CD23 (StemCell Technologies, Vancouver, Canada) and negative immunomagnetic cell separation technique. The resultant cell suspensions routinely contained at least 98% macrophages as assessed by flow cytometry using a FITC-labeled rat anti-mouse F4/80 mAb (Harlan Bioproducts for Science) reactive with murine macrophages (22). Thioglycollate-elicited macrophages from SCID mice were isolated following the above protocol after in vivo immunodepletion of NK cells by i.v. injection of 100 μg per mouse of both polyinosinic-polycytidylic acid and rabbit anti-asialo-GM1 antisera 3 days before cell isolation (23).

Purified peritoneal macrophages were resuspended in RPMI 1640 culture medium containing 10% FBS (Sigma; endotoxin level ≤1.0 ng/ml) and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Approximately 1.0 × 10^6 macrophages were dispersed in each well of a 24-well tissue culture plate (Costar, Cambridge, MA) and incubated in a humidified atmosphere of 5% CO₂ for 30 min at 37°C. Macrophages were either directly stimulated with indicated concentrations of LPS from E. coli O111:B4 for 24 h to assess the primary LPS-induced responses or, in parallel experiments, were first LPS reprogrammed by pretreatment with substimulatory LPS concentrations in a range of 0.01–10 ng/ml for 6 h and then challenged with optimally effective LPS doses of 100 ng/ml for 24 h. Culture supernatants were collected at the end of incubation period and frozen at −70°C for further determination of cytokine and NO concentrations.

Flow cytometry

Flow cytometry analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA). Approximately 1 × 10^6 cells were washed with PBS and resuspended in 100 μl of PBS containing 2% BSA and 0.05% sodium azide. Non-specific binding of Abs was blocked with purified anti-mouse CD16/CD32 mAb (Fc block; BD Pharmingen). Staining was performed in the dark at 4°C for 20 min using the optimal concentrations of FITC-labeled DX5 mAb determined in pilot experiments. After staining, cells were washed twice with cold PBS/NaCl and fixed with 1.0% (w/v) paraformaldehyde for 30 min at 4°C. Non-specific binding was determined by using isotype-matching FITC-labeled Abs (anti-KLH mAb; BD Pharmingen). Dead cells were labeled with 10 μg/ml propidium iodide (Sigma). Cell populations were gated using forward and side scatter characteristics to exclude debris and/or cell aggregates and forward scatter characteristics to assess the purity of the population. For each measurement at least 10^4 cells were acquired.

Cytokine and NO assays

TNF-α bioactivity in culture supernatants was determined by a cytotoxicity assay on actinomycin D-treated L929 mouse fibroblasts using mouse rTNF-α (4 × 10^3 U/mg; Genzyme, Cambridge, MA) as an internal standard (21). ELISA based on mouse IL-1β Douset (Genzyme; sensitivity 20 pg/ml) was used to measure IL-1β ELISA for determination of IL-12 p70 heterodimer concentrations was performed using a pair of monoclonal anti-IL-12 Abs (19361V and 20171D; BD Pharmingen) and mouse rIL-12 (5 × 10^3 U/mg; Genzyme) as a standard. The sensitivity of IL-12-specific ELISA was ≈20 pg/ml. Recombinant mouse IFN-γ (19301T; BD Pharmingen), purified rat anti-mouse IFN-γ capture Ab (18181D; BD Pharmingen), and biotinylated rat anti-mouse IFN-γ detection Ab (18112D; BD Pharmingen) were used for detection of IFN-γ. The sensitivity of IFN-γ-specific ELISA was at least 20 pg/ml. NO was measured as nitrite, a stable product of NO decay, using the Griess reagent as described previously (24).

Statistical analysis

Whenever applicable, the results were statistically analyzed using StatView software package (Abacus Concepts, Berkeley, CA). All data are presented as mean ± SEM. Changes in the levels of cytokines and NO over the baseline were analyzed by ANOVA. The differences were considered significant at p < 0.05.

Results

Role of IFN-γ in LPS-induced responses of intact and LPS-reprogrammed macrophages

To determine the potential contribution of IFN-γ to LPS-induced activation of resting and LPS-reprogrammed macrophages, we compared cytokine and NO production by LPS-stimulated thiglycollate-elicited macrophages isolated from wild-type C57BL/6 mice and congenic IFN-γ−/− mice with a targeted disruption of IFN-γ gene. To assess the primary LPS-induced responses, purified macrophages were stimulated in vitro with various concentrations of E. coli O111:B4 LPS ranging from 1.0 pg/ml to 100 ng/ml for 24 h. After stimulation, the culture supernatants were collected and TNF-α, IL-12, and NO concentrations were measured. As anticipated, macrophages isolated from wild-type C57BL/6 mice responded in a dose-dependent manner over the range of 1–100 ng/ml of LPS as assessed by TNF-α, IL-12, and NO production (Fig. 1A, B, and C, respectively). In contrast, LPS-induced TNF-α production by macrophages from IFN-γ−/− mice was markedly reduced (Fig 1A), and neither IL-12 nor NO responses were essentially distinguishable from the corresponding background levels produced by resting unstimulated IFN-γ−/− macrophages (Fig. 1, B and C, respectively).

1 Abbreviations used in this paper: ICE, IL-1β-converting enzyme; KO, knockout; L-NMMA, N6-monomethyl-L-arginine.
However, hyporesponsiveness of IFN-$\gamma^{-/-}$ macrophages to LPS could be fully restored by an exogenous mouse rIFN-$\gamma$. Thus, IFN-$\gamma^{-/-}$ macrophages challenged with LPS in the presence of 1.0 U/ml rIFN-$\gamma$ manifested a potent secretion of both TNF-$\alpha$ and IL-12 (Fig. 1, A and B, respectively). However, LPS-induced NO production by IFN-$\gamma^{-/-}$ macrophages in the presence of exogenous rIFN-$\gamma$ was rather strikingly potentiated as compared with the IFN-$\gamma^{+/+}$ macrophage responses to LPS alone (Fig. 1C).

To address the question whether IFN-$\gamma$ is equivalently important in LPS-dependent reprogramming effects, we analyzed TNF-$\alpha$, IL-12, and NO production by IFN-$\gamma^{-/-}$ macrophages after pretreatment with substimulatory LPS doses in a range of concentrations from 10 pg/ml to 10 ng/ml and subsequent challenge with LPS. The reprogramming concentrations of LPS, when used for a direct stimulation of IFN-$\gamma^{-/-}$ macrophages for 24 h, failed to induce significant cytokine or NO responses as illustrated by the data presented in Fig. 1. Rather remarkably, however, LPS-dependent reprogramming resulted in a 30- to 50-fold increase in TNF-$\alpha$ production by IFN-$\gamma^{-/-}$ macrophages as compared with the optimal LPS-induced TNF-$\alpha$ responses in IFN-$\gamma^{+/+}$ macrophages in the presence of rIFN-$\gamma$ (Figs. 1A and 2A). Likewise, reprogramming with LPS substantially potentiated IL-12 responses in IFN-$\gamma^{-/-}$ macrophages, although to a lesser extent than does exogenously added rIFN-$\gamma$ (Fig. 2B). In contrast, NO production by the reprogrammed IFN-$\gamma^{-/-}$ macrophages remained at the background level after 24-h challenge with LPS, thereby strongly indicating the requirement of IFN-$\gamma$ for potent induction of NO responses (Fig. 2C).

**Reciprocal role of IL-10 and IL-12 in modulation of LPS-reprogramming effects**

Because the data presented above suggest an IFN-$\gamma$-independent pathway(s) in modulation of cytokine responses in macrophages, experiments were undertaken to further explore this concept by assessing the potential contribution of autocrine control mechanisms mediated by such immunoregulatory cytokines as IL-10 and IL-12. In these experiments, we compared TNF-$\alpha$ responses of macrophages isolated from IL-10-deficient (IL-10$^{-/-}$), IL-12-deficient (IL-12$^{-/-}$), and the parental wild-type C57BL/6 mice after LPS-dependent reprogramming and subsequent challenge with LPS (Fig. 3). The data presented in Fig. 3 show that LPS-dependent reprogramming of macrophages isolated from IL-10$^{-/-}$ mice with a targeted disruption of IL-10 gene strongly potentiated TNF-$\alpha$ responses by the cells after LPS challenge as compared with LPS-induced TNF-$\alpha$ production by the reprogrammed macrophages from the parental C57BL/6 mice. In contrast, TNF-$\alpha$ responses by the reprogrammed and LPS-challenged macrophages isolated from IL-12$^{-/-}$ mice were substantially attenuated as compared with the control cytokine levels produced by IL-12$^{-/-}$ macrophages upon identical experimental conditions (Fig. 3). Interestingly, the profile of NO secretion modulated by LPS-dependent reprogramming was not significantly altered in macrophages isolated from either IL-10$^{-/-}$ or IL-12$^{-/-}$ mice (data not shown). Collectively, these findings strongly indicate the negative and positive regulatory effects of IL-10 and IL-12, respectively, on LPS-dependent reprogramming mechanism(s) controlling the modulation of TNF-$\alpha$ potential in macrophages.

**Role of autocrine pathways in reprogramming of macrophages inflammatory responses**

Because both cytokines can be produced by activated macrophages, IL-10- and IL-12-dependent autocrine cytokine regulatory circuits may well be operative in modulation of the magnitude and repertoire of inflammatory responses in LPS-reprogrammed macrophages. Although, in the previous experiments, we used highly purified peritoneal macrophages, these experimental conditions do not exclude completely the potential regulatory effects of other immunocompetent cells and/or their products on in vivo modulation of macrophage inflammatory responses subsequently analyzed in our studies in vitro. Therefore, to provide additional evidence...

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**FIGURE 1.** IFN-$\gamma$-dependent regulation of LPS-induced responses in resting macrophages. Immunomagnetically selected peritoneal macrophages from wild-type (IFN-$\gamma^{+/+}$) (○) and IFN-$\gamma$-deficient (IFN-$\gamma^{-/-}$) C57BL/6 inbred mice (circles) were stimulated with indicated concentrations of O111:B4 LPS for 24 h in the absence (●) or presence of 1.0 U/ml mouse rIFN-$\gamma$ (▲). After stimulation, cell culture supernatants were collected and TNF-$\alpha$ (A), IL-12 (B), and NO (C) responses were determined following the procedures described in Materials and Methods. The data are mean ± SEM. One of three independent experiments is shown. *, p < 0.05 vs baseline cytokine and NO levels in unstimulated macrophages.
that macrophage-derived IL-10 and IL-12 and their autocrine effects primarily contribute to the observed phenomenon of LPS-dependent reprogramming, we evaluated TNF-α and NO responses of macrophages immunomagnetically isolated from SCID mice. Although macrophages from SCID mice maintain a broad range of specific activities, T and B cells are characterized by functional incompetence. To exclude the potential IFN-γ-dependent modulation of macrophage inflammatory responses by NK cells, we performed experiments in vivo using IFN-γ−/− mice.

**FIGURE 2.** TNF-α, IL-12, and NO responses in LPS-reprogrammed IFN-γ−/− macrophages. Left, Immunomagnetically enriched peritoneal macrophages from IFN-γ−/− mice were reprogrammed in a dose-dependent manner with indicated substimulatory concentrations of LPS, washed with HBSS, and then challenged with 100 ng/ml LPS for 24 h. Cell culture supernatants were collected and the levels of TNF-α (A), IL-12 (B), and NO (C) induced in LPS-reprogrammed IFN-γ−/− macrophages were determined as described in Materials and Methods. *p < 0.05 vs cytokine and NO levels in unprimed macrophages directly stimulated with LPS. Right, In parallel experiments, purified resting IFN-γ−/− macrophages were directly stimulated with 100 ng/ml LPS for 24 h in the absence or presence of 1.0 U/ml mouse rIFN-γ. The data shown are mean ± SEM of triplicate measurements from one representative experiment of three performed. *p < 0.05 vs the levels of cytokines or NO in LPS-stimulated macrophages from the parental wild-type mice.

**FIGURE 3.** Differential regulation of cytokine responses in LPS-reprogrammed macrophages from IL-10 KO and IL-12 KO mice. Isolated macrophages were LPS-reprogrammed by pretreatment with indicated substimulatory concentrations of O111:B4 LPS for 6 h and then stimulated with 100 ng/ml LPS for 24 h. Cell culture supernatants were collected and levels of TNF-α (A), IL-10 (B), and IL-12 (C) produced by macrophages isolated from the parental wild-type C57BL/6 mice (○), IL-10−/− mice (■), and IL-12−/− mice (▲) were measured as described in Materials and Methods. The data represent mean ± SEM of triplicate determinations from one representative experiment of three performed. *p < 0.05 vs the corresponding cytokine concentrations produced by LPS-reprogrammed macrophages from the parental wild-type mice.
cells, SCID mice were immunologically depleted of NK cells by i.v. injection of both polyinosinic-polycytidylic acid and rabbit anti-asialo-GM1 antiserum as confirmed by flow cytometry (Fig. 4).

By using the standard methodologies, we analyzed LPS-induced cytokine and NO responses of the reprogrammed macrophages isolated from NK cell-depleted SCID mice. The results of these studies summarized in Fig. 5 strongly suggest that in a range of LPS concentrations of 10–500 pg/ml and 0.5–10 ng/ml LPS-dependent reprogramming modulates either predominant TNF-α or NO phenotype of response, respectively. Therefore, LPS-reprogramming effects on macrophages can be observed in the absence of potential regulatory signals generated by other immunocompetent cells. These findings suggest that autocrine regulatory mechanisms and pathways, at least in part, differentially regulate the magnitude and profile of macrophage inflammatory response.

Role of IL-18, IL-1β, PGs, and NO in modulation of LPS-reprogramming effects

In addition to IL-10 and IL-12, other macrophage-derived inflammatory mediators including IL-1β, IFN-γ-inducing factor, or IL-18, and PGs can potentially mediate the observed differential cytokine responses in LPS-reprogrammed macrophages. Because both IL-1 and IL-18 knockout (KO) mice were not available for these studies, we sought to find an alternative experimental approach to evaluate the prospective involvement of IL-1 and/or IL-18 in regulation of LPS-dependent reprogramming effects in macrophages. Because both IL-1β and IL-18 are initially synthesized by the cells as inactive precursors, pro-IL-1β and pro-IL-18, which subsequently are converted into secreted cytokines by ICE or caspase-1, we used a highly selective inhibitor of ICE, Ac-Tyr-Val-Ala-Asp-aldehyde, to block completely LPS-induced secretion of IL-1β and IL-18 by macrophages (25, 26). In pilot studies, it was found that 20 μM of Ac-Tyr-Val-Ala-Asp-aldehyde completely block the secretion of IL-1β by stimulated peritoneal macrophages (compare 1250 pg/ml IL-1β vs 187 pg/ml in the absence and presence of ICE inhibitor, respectively) under our specific experimental conditions. Using this effective concentration of ICE inhibitor, we demonstrated that Ac-Tyr-Val-Ala-Asp-aldehyde, when added to macrophage culture during the LPS-reprogramming stage, does not affect the outcome of unique polarization of TNF-α and NO responses in LPS-reprogrammed macrophages (Fig. 6).

Likewise, 20 μg/ml of the neutralizing anti-mouse IL-18 Abs added to the cells during the reprogramming step were unable to affect differential modulation of TNF-α and NO macrophage responses (Fig. 6).

Because PGs, and PGE2, in particular, are known to be important inhibitors of TNF-α production (27), we sought to evaluate potential contribution of these mediators to the regulatory mechanisms governing the LPS-dependent reprogramming effects. The data shown in Fig. 6 strongly suggest that PGs are unlikely to be involved in selective modulation of a biphasic profile of TNF-α response because 10−4 M of a cyclooxygenase inhibitor ibuprofen, which completely blocked PGE2 secretion by LPS-stimulated macrophages (data not shown), did not restore the marginal TNF-α production by macrophages reprogrammed for NO phenotype of response.

Furthermore, recent findings strongly implicate NO in inhibition of NF-κB-dependent transcriptional activation of various genes that often encode proinflammatory mediator and cytokines. It was shown that NO induces S-nitrosylation of the reactive thiol groups of cysteine residues of NF-κB/Rel proteins that ultimately prevent NF-κB from binding to the cognate sites in the promoter regions of TNF-α gene (28). To explore whether macrophage-derived NO contributes to a biphasic profile of TNF-α production, we investigated the effects of L-NMMA, an inhibitor of inducible NO synthase, on TNF-α responses of LPS-reprogrammed macrophages. Our data summarized in Fig. 6 would rather support the conclusion that the observed inhibition of TNF-α responses is not mediated by excessive production of NO manifested by macrophages reprogrammed for predominant NO phenotype of response.
The data obtained by using macrophages isolated from IFN-γ−/− mice provided further support for the concept of important immunoregulatory role of IFN-γ in LPS-induced cytokine and NO responses in macrophages (Fig. 1). Thus, marginal production of TNF-α, IL-12, and NO by LPS-activated IFN-γ−/− macrophages was strongly up-regulated in the presence of exogenous rIFN-γ. However, when LPS-hyporesponsive IFN-γ−/− macrophages were pretreated with substimulatory concentrations of LPS and subsequently challenged with LPS, these LPS-reprogrammed macrophages manifested the capacity to produce robust TNF-α and IL-12 cytokine responses (Fig. 2). Modulation of such potent cytokine responses upon experimental conditions that completely exclude the regulatory effects of IFN-γ would strongly suggest the existence of an alternative IFN-γ-independent mechanism(s) controlling the inflammatory cytokine potential in macrophages. However, LPS-reprogrammed IFN-γ−/− macrophages continue to exhibit hyporesponsive in regard to LPS-induced activation of inducible NO synthase and production of NO (Fig. 2C). These findings further support the concept that cytokine and NO responses in macrophages are not uniformly controlled by identical environmental signals including bacterial LPS (21, 29).

To ascertain the functional role of IL-10 and IL-12 in IFN-γ-independent regulation of LPS-reprogramming effects, we used macrophages isolated from KO mice with a targeted disruption of either IL-10 or IL-12 gene. The data summarized in Fig. 3 strongly indicate that neither endogenously produced IL-10 nor IL-12 is solely responsible for the observed phenomenon of LPS-reprogrammed TNF-α responses, although these responses were strongly potentiated in IL-10−/− macrophages and substantially attenuated, but not completely inhibited, in IL-12−/− macrophages. Because IFN-γ can be produced by macrophages stimulated with LPS in the presence of IL-18 and IL-12 (20), we next investigated the functional role of endogenously produced IL-18 in regulation of LPS-reprogramming effects in macrophages. Neutralization of endogenous IL-18 with 20 μg/ml anti-IL-18 Abs, which inhibit >90% of the costimulatory activity of 1 ng/ml rIL-18 on IFN-γ production by spleen lymphocytes stimulated with 0.75 μg/ml Con A, did not affect the modulation of either TNF-α or NO response in LPS-reprogrammed macrophages (Fig. 6). In addition, LPS-dependent reprogramming of these response were not significantly changed in the presence of an ICE inhibitor blocking the conversion of both pro-IL-1β and pro-IL-18 cytokine precursors into biologically active cytokines secreted by the cells (25, 26). Furthermore, it appears that endogenously produced PGs are not directly involved in modulation of biphasic TNF-α responses in LPS-reprogrammed macrophages (Fig. 6), although these mediators are known to inhibit TNF-α production (27). Collectively, our data would reasonably support a conclusion that among different macrophage-derived cytokines and other inflammatory mediators, the effects of which were analyzed in the present study, only endogenously produced IL-10 and IL-12 are involved in IFN-γ-independent up-regulation of TNF-α and IL-12 cytokine responses in LPS-reprogrammed macrophages.

Using macrophages isolated from SCID mice with immunologically depleted NK cells, we were able to provide strong support for our experimental hypothesis that LPS-dependent reprogramming effects on TNF-α and NO responses in macrophages are primarily controlled by autocrine regulatory mechanisms (21). As an extension of this concept, these reprogramming mechanisms can be instrumental in regulation of the adjuvant properties of macrophages defined as the capacity of these cells to produce immunoregulatory cytokines and generate other accessory or costimulatory signals implicated in the development of Ag-specific

**FIGURE 6.** Potential role of macrophage-derived IL-1β, IL-18, and PGs in autocrine modulation of LPS-reprogramming effects in macrophages. To modulated TNF-α (A) or NO (B) phenotypic response, macrophages isolated from normal C57BL/6 mice were reprogrammed by 6-h pretreatment with either 0.5 or 5 ng/ml O111:B4 LPS, respectively. LPS-dependent reprogramming were conducted in the absence of other additives (control) or in the presence of either 20 μM of ICE inhibitor, 20 μg/ml rabbit anti-IL-18 or control nonimmune rabbit IgG, 10−4 M of ibuprofen, or 1 mM of L-NMMA. After reprogramming, macrophages were washed several times with HBSS and then stimulated for 24 h with 100 ng/ml LPS. TNF-α and NO levels in culture supernatants were assessed as described in *Material and Methods*. The data represent the mean ± SEM of triplicate measurements from one representative experiment of four performed.

**Discussion**

Macrophages are unique cells of innate immunity with an impressive repertoire of potential inflammatory responses that enable these cells to continuously conduct immunosurveillance in health and disease. Depending on the tissue location and surrounding microenvironment, macrophages are capable of activating one of the numerous immunologic programs that are ultimately associated with the acquisition of a specific phenotypic response accommodated by changes in gene expression and cellular metabolism.

We have recently hypothesized that, at the early stage of infection, macrophages can be selectively reprogrammed for a specific phenotype of immune responses by exposure to substimulatory concentrations of different microbial components and products including LPS (21). In support of this fundamental concept, we have described the phenomenon of LPS-dependent reprogramming of macrophage inflammatory responses that are, at least in part, controlled by a reciprocal modulation of pro- and anti-inflammatory cytokine responses in the cells. Because macrophages are capable of producing proinflammatory cytokines such as IL-1β, IL-12, IL-18, and IFN-γ, which can restrain the anti-inflammatory effects of macrophage-derived IL-10, we have extended our previous studies to define specific role of these cytokines in LPS-reprogramming effects by using macrophages isolated from mice with targeted disruption of cytokine genes.
immune responses. Activated by such reprogramming mechanisms, macrophages may well convey instructive signals to other cells of the immune system and provide fundamentally important “early assessment” of the invading pathogen to elicit an appropriate effector mechanism of acquired immunity. In this regard, it also appears that the lack of LPS-dependent cytokine-driven immune responses in LPS-hyporesponsive C3H/HeJ mice may, at least in part, account for extremely high sensitivity of these mice to Gram-negative infections (30). Further investigation of the integrity and efficiency of regulatory mechanisms of innate immunity is important, because better understanding of these processes provides fundamental approaches for the development of new therapeutic strategies in immunological correction of altered immune responses.

References


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