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Intrinsic Defects in Macrophage IL-12 Production Associated with Immune Dysfunction in the MRL/++ and New Zealand Black/White F_1 Lupus-Prone Mice and the *Leishmania major*-Susceptible BALB/c Strain[1]

David G. Alleva,[2] Steven B. Kaser, and David I. Beller[3]

We have demonstrated that macrophages (Mϕ) from young, prediseased, lupus-prone MRL/++ and New Zealand Black/White F_1 mice display defective production of TNF-α, IL-1, and IL-6, but normal production of IL-10. In an attempt to determine the potential functional implications of this phenotype for autoimmunity, we demonstrate here that endotoxin-activated Mϕ from these lupus-prone mice showed dramatically reduced expression of IL-12, a cytokine essential for Th1 responses that may be defective during lupus. IL-12 production was also reduced by Mϕ from the control BALB/c strain, compatible with the concept that a genetically programmed deficit in IL-12 levels may underlie the IL-4-dominated BALB/c response to infection by the parasite *Leishmania major*. Although both IL-12 and TNF-α expression defects by Mϕ from lupus-prone strains are expressed rapidly after activation, treatment with each cytokine demonstrated that only TNF-α contributes to the subsequent dysregulation of Mϕ IL-1 and IL-6 expression in these strains, and that the reduced autocrine activity of defective IL-12 or TNF-α levels was not causal to each other. Although the intrinsic defect in IL-12 expression by lupus-prone and BALB/c Mϕ may lead to defective Th1 responses, these Mϕ responded to the Th1-derived cytokine, IFN-γ, in a normal fashion suggesting a defective role in the induction, rather than the propagation, of Th1 responses in these mice. Our finding of a conserved intrinsic defect in IL-12 production by Mϕ from the two principal mouse models of multigenic lupus provides insight into how excessive humoral responses may develop, and perhaps be prevented, in systemic autoimmune disease. *The Journal of Immunology,* **1998, 161: 6878–6884.**

It is widely held that self-reactive lymphocytes are a normal component of the immune system, and it is the inability to limit the growth and function of these autoreactive cells that characterizes both organ-specific and systemic autoimmune diseases (1). In particular, systemic lupus erythematosus (SLE)[4] is characterized by excessive B cell activity and abnormally high serum autoantibody levels. This leads to the deposition of Ab and immune complex in many tissues, and ultimately, to severe organ or tissue pathology, including vasculitis and glomerulonephritis (2). Unlike organ-specific B cell-mediated autoimmune diseases like myesthenia gravis, or Graves' disease, the breadth of the Ab repertoire that develops during lupus suggests that the nature of the defects in immune function that trigger disease are likely to have global implications (e.g., involving regulatory molecules such as cytokines) rather than being finely focused (e.g., dependent upon selected B or T cell receptors or MHC polymorphisms).

Fundamental to understanding the initiation of autoimmune disease is the determination of why the control of autoreactive lymphocyte function is not established. One critical issue to address is the extent to which lymphocyte defects are truly intrinsic, or, alternatively, result secondarily from defects in the function of APCs or other regulatory cell populations. Evidence exists for both of these positions. Evidence supporting intrinsic B cell defects has come primarily from studies using MRL/*lpr* mice (3, 4). Because these mice display aberrant, nonfunctional Fas proteins, all cell populations in MRL/*lpr* mice whose growth and function are regulated by Fas-induced apoptosis are likely to be affected. The extent to which mice bearing the *lpr* mutation provide insight into the basis of multigenic human autoimmunity remains to be determined. The demonstration of B cell hyperactivity in young New Zealand Black/White (NZB/W) F_1 and BxSB lupus-prone mice, or the transfer of T cell-depleted bone marrow from these mice into SCID mice (5, 6), has suggested that B cells from these multigenic lupus models may also have intrinsic defects in function. While such experiments emphasize the early development of aberrant B cell function in lupus, they do not substantiate that defective function is intrinsically programmed within the B cell, and the importance of accessory cells in permitting expression of defective B cell function has been demonstrated (7).

Studies focusing on the function of regulatory cells, such as macrophages (Mϕ) (8–15), from the best-studied murine lupus models, MRL/++ and NZB/W F_1, have revealed dramatic defects in production of the proinflammatory cytokines TNF-α (12, 15), IL-1 (8–10, 15), and IL-6 (15), but not the anti-inflammatory cytokine IL-10 (15). Of these, aberrant IL-1 expression has been shown to be truly intrinsic, i.e., independent of the contribution of other cell types (9, 10). It is noteworthy that the onset of defective

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[4] Abbreviations used in this paper: SLE, systemic lupus erythematosus; NZB/W, New Zealand Black/White; Mϕ, macrophages; rMu, recombinant murine.

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production of the individual proinflammatory cytokines occurs sequentially, and moreover, that the reduced autocrine stimulation caused by the TNF-α defect appears to be responsible for the subsequent IL-1 and IL-6 defects (15). Nevertheless, each of these events is triggered in and controlled by Mϕ alone and is thus intrinsic to the Mϕ. These studies have led us to question the potential functional contribution of Mϕ to B cell hyperactivity in these mice. This contribution could be both direct, on the B cell itself, and indirect, via modulation of T cell function. To address these issues, we first extended our characterization of Mϕ from MRL/++ and NZB/W F_1 mice to include the immunomodulatory cytokine, IL-12, which is mainly produced by Mϕ and is critical for directing development of the Th1 responses (16) that may be defective in lupus-prone mice (17). Our results show that production of IL-12 is dramatically reduced in Mϕ from both MRL/++ and NZB/W F_1 mice, which appears to be similar in nature to that of TNF-α but not the subsequent IL-1 and IL-6 defects. In addition, Mϕ IL-12 production in response to the Th1-derived cytokine, IFN-γ, was normal in these lupus-prone strains, suggesting a defect in the initiation, but not the propagation, of Th1 responses in the disease process. This study provides additional insights into the nature of the cytokine defects that uniquely characterize the two principal models of multigenic lupus and suggests that the cytokine-dependent regulation of the Th1 subset by the cells of the innate immune system may be an important event in the establishment of lupus.

Materials and Methods

Animals

Four-week-old BALB/c, A/J, C57BL/6, C57BL/10, DBA/2, C3H/OuJ, MRL/++, and NZB/W F_1 male mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained for 1 wk after arrival in a pressurized (one-way flow) room.

Reagents

Recombinant murine (rMu)TNF-α (10^7 U/mg; cat. no. 19321T) was purchased from PharMingen (San Diego, CA), rMuIFN-γ (10^7 U/mg) was kindly provided by Genentech (South San Francisco, CA), and rMuIL-12 (3×10^6 U/mg) was a gift from Dr. Victor H. Van Cleave (Genetics Institute, Cambridge, MA). LPS (*Escherichia coli*: 0111:B4; Sigma, St. Louis, MO) was stored at −70°C (2 mg/ml in H_2O) and diluted immediately before use. RPMI 1640 medium with glutamine (cat. no. 12-702F) was supplemented with 0.5% HEPES (cat. no. 17-737), 1% penicillin/streptomycin solution (cat. no. 17-602E), and 5% FBS (all components of medium from BioWhittaker, Walkersville, MD) and was used for culturing Mϕ. HBSS (BioWhittaker) was used for cell washing.

Mϕ isolation and culturing

Peritoneal exudate Mϕ were obtained by peritoneal lavage with cold HBSS 4 days after a 2 ml i.p. injection of 4% thioglycollate broth (Remel, Windsor, CT; cat. no. 07178). Cells were pooled from at least three mice per strain, washed, resuspended in fresh medium, and seeded at 10^5 cells in 100 μl per well of 96-well flat bottom tissue culture-treated plates (Costar, Cambridge, MA; cat. no. 3596). Cells were incubated for 2 h at 37°C, 5% CO_2, in a humidified chamber to allow Mϕ to adhere and spread. Nonadherent cells were removed by adding 200 μl of warmed HBSS to each well, resuspending nonadherent cells by moderately tapping the plate, and flicking the plate to discard the nonadherent cells. This washing was performed three times, after which 50 μl of medium was immediately added to each well. Remaining cells were >98% Mϕ, as assessed by morphologic examination and nonspecific esterase staining. Although ~85% of the total exudate cell population were adherent Mϕ within each strain, to ensure that equal numbers of adherent Mϕ among strains remained after washing, nonadherent cells from washes of single wells from each strain were routinely counted and showed no significant differences among strains. Cytokines, Abs, LPS, or medium were added to each well to yield a final volume of 200 μl. Conditioned medium was collected in a sequential fashion (e.g., 0–16, 16–24, 24–36 h), with change of medium and fresh LPS along with other factors at each time point, and stored at −20°C for assessment of cytokines. This method of conditioned medium collection has the advantage of permitting measurement of cytokine levels independent of levels produced during an earlier period, a feature that contributes to accurate assessment of changes in the kinetic pattern of cytokine production. The three culturing periods used for the kinetic assessment of cytokine correlate to an early, intermediate, and late period that collectively span the entire time during which most of the cytokine is produced. For example, >90% of the total amount of IL-12 is produced within 24 h, and the rest is produced within 24–36 h (see Fig. 2; see Ref. 15 for kinetics of other cytokines).

Assessment of cytokines

IL-12 levels in Mϕ-conditioned medium were assessed by a MuIL-12-specific ELISA, a gift from Dr. Victor H. Van Cleave. The general cytokine ELISA protocol developed by PharMingen was used. Briefly, two rabbit polyclonal anti-MuIL-12 Abs preparations, R03B03 (3 μg/ml) and B03B02 (biotinylated, 1:500 dilution), that were prepared by immunizing rabbits with the IL-12 heterodimer (p70) were used for capture and detection, respectively, along with avidin-labeled peroxidase (Sigma, cat. no. A-3151) and 2,2′-azino-bis (3-ethyl benzthiazoline-6-sulfonic acid) solution (Sigma, cat. no. A-1888) for development. One hundred microliters of undiluted Mϕ-derived conditioned medium was added to the ELISA, and rMuIL-12 was used to generate a standard curve. IL-6 and IL-10 levels in Mϕ supernatants were assessed by ELISA (mAb from PharMingen; anti-IL-6 mAb, cat. nos. 18071D and 18082D, anti-IL-10 mAb, cat. nos. 18141D and 18152D). IL-1 bioactivity in Mϕ lysates was measured in the D10.G4 bioassay as previously described (15, 18), and TNF-α levels in Mϕ conditioned medium were assessed using the WEHI-164 bioassay as previously described (15, 19). One unit of cytokine activity in bioassay is defined as the amount of cytokine that caused half maximum cell viability as assessed with Alamar Blue solution (AccuMed International, Westlake, OH; cat. no. 00–100).

Statistical analysis of data

All means and SE in figures were calculated from triplicate values and the Student's *t* test was used to compare mean values.

Results

Aberrant IL-12 production by Mϕ from MRL/++ and NZB/W F_1 mice

To evaluate one possible mechanism for aberrant Mϕ cytokine production contributing to the dysregulation of lymphocyte function in lupus, we investigated whether Mϕ from lupus-prone strains aberrantly produced IL-12. This cytokine is known to play a dominant role in the appropriate development of Th1 cells, in establishing the balance of Th1 and Th2 populations in normal individuals, and in triggering the divergence from this balance that appears to be a critical component of the development of autoimmune disease (16, 17). Peritoneal Mϕ were obtained from young (4-wk-old), prediseased, male MRL/++ and NZB/W F_1 mice and from five age-matched control murine strains, BALB/c, A/J, C57BL/6, DBA/2, and C3H/OUJ. Mϕ were activated with LPS for 16 h, and culture-conditioned medium was assessed for IL-12 levels by ELISA (Fig. 1). The data reveal that Mϕ from MRL/++ and NZB/W F_1 strains are dramatically impaired and produce 10- to 20-fold less IL-12 than all control strains, except BALB/c. Activation of Mϕ with other doses of LPS (i.e., 10 ng/ml and 1000 ng/ml) resulted in defects of similar magnitude in Mϕ from the lupus-prone strains (data not shown). Interestingly, BALB/c Mϕ produce notably less IL-12 (~4-fold less) than Mϕ from the other control strains. Thus, the BALB/c strain, which is known to have defective development of a protective Th1 type response during some parasitic infections (i.e., *Leishmania major*; reviewed in Ref. 20), is here shown to have substantially impaired production of the Th1-inducing cytokine, IL-12. The association in BALB/c mice of low IL-12 levels with restrictions in selected Th2-dependent immune responsiveness suggests that the similar, if not more pronounced, IL-12 defect in the MRL/++ and NZB/W F_1 strains (in nine experiments, MRL/++ and NZB/W F_1 Mϕ produced IL-12 levels at an average of 62% ± 10% ($p < 0.01$) and 70% ± 12%

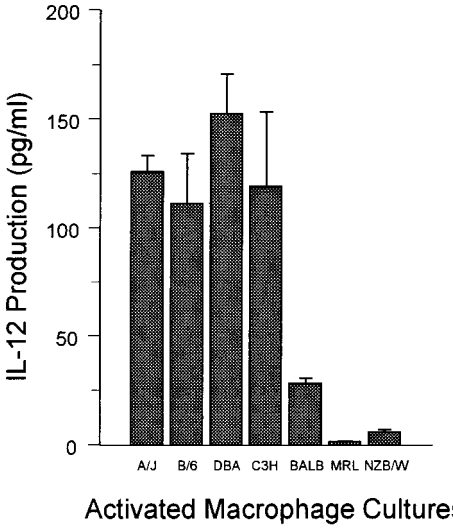

FIGURE 1. Defective IL-12 expression in Mϕ from lupus-prone mice. Thioglycollate-elicited peritoneal Mϕ (10^5) from control (BALB/c, A/J, C57BL/6, DBA, and C3H/OuJ) and lupus-prone (MRL/++ and NZB/W F_1) murine strains were activated with 100 ng/ml LPS and incubated for 16 h, at which time conditioned medium was removed and stored at −20°C until assessed for IL-12 levels. Each bar represents the mean and SE of triplicate values, and these results are representative of four experiments that had similar results. No IL-12 was detected in unstimulated Mϕ cultures.

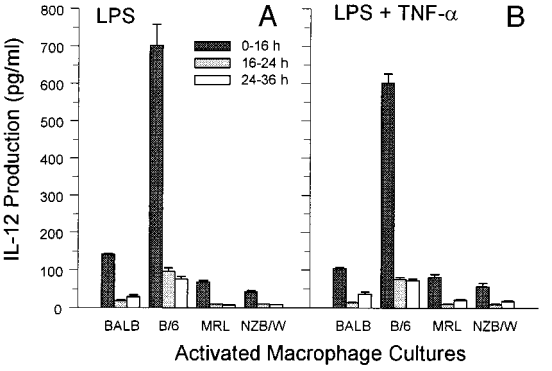

FIGURE 2. TNF-α does not regulate defective IL-12 expression in Mϕ from lupus-prone mice. Thioglycollate-elicited peritoneal Mϕ (10^5) from control (BALB/c and C57BL/6) and lupus-prone (MRL/++ and NZB/W F_1) murine strains were activated with 100 ng/ml LPS in the absence (*A*) or presence of 1000 U/ml TNF-α (*B*) and incubated for three sequential intervals in which conditioned medium was removed and cultures replenished with fresh medium and stimuli. Conditioned medium from each culture interval was assessed for IL-12 levels, and each bar represents the mean and SE of triplicate values. Results are representative of four experiments that had similar results.

($p < 0.01$), respectively, of those of the BALB/c Mϕ during the first period) may well have physiological relevance to the immune dysregulation in these strains.

To characterize the kinetics of cytokine production, IL-12 levels were next assessed in conditioned medium collected during three sequential intervals that represent early (0–16 h), intermediate (16–24 h), and late (24–36 h) periods of IL-12 production, as described in *Materials and Methods*. Mϕ from C57BL/6 mice were selected as a representative control strain because they routinely produced levels of IL-12 close to the median value for normal strains. Mϕ from MRL/++ and NZB/W F_1 mice consistently displayed at least a 10-fold reduction in IL-12 production relative to C57BL/6, regardless of the period of evaluation (Fig. 2*A*). BALB/c Mϕ, as noted earlier, produced substantially less IL-12 than Mϕ from any other normal strain. Defective IL-12 expression by Mϕ from lupus-prone strains is not a consequence of a reduction in either viability or metabolic activity and does not reflect a broad dysregulation of Mϕ function inasmuch as viability, protein synthesis, and induction of LFA-1 and IL-10 all do not significantly vary between Mϕ from control and lupus-prone strains (Refs. 15 and 21, and data not shown). These results show that the defective Mϕ cytokine production profile in the MRL/++ and NZB/W F_1 strains, originally defined as encompassing IL-1, IL-6, and TNF-α, but not IL-10 (15), can now be extended to include IL-12 as well.

Lupus-prone Mϕ defects in IL-12 and TNF-α production do not impact each other

Defective TNF-α production by Mϕ from lupus-prone mice is manifested soon after stimulation (0–12 h), and clearly before defective expression of IL-6 (12–24 h) or IL-1 (48 h) (15). In fact, compromised autocrine stimulation by TNF-α in MRL/++ and NZB/W F_1 Mϕ appears to cause the subsequent defects in IL-1 and IL-6 production (15). Therefore, we addressed whether the early TNF-α defect might contribute in a similar manner to the dramatically reduced IL-12 expression in these Mϕ or, conversely, whether defective IL-12 might regulate TNF-α expression. Mϕ from control and lupus-prone mice were activated with LPS in the presence or absence of a physiological concentration of TNF-α (1000 U/ml, a level routinely produced by control Mϕ under these conditions (15)). Conditioned medium was collected at 16 h and assessed for IL-12 (Fig. 2, *A* and *B*). TNF-α did not stimulate LPS-activated IL-12 production by Mϕ from any strain during any interval, and, thus, it is unlikely that the defective TNF-α production characteristic of Mϕ from lupus-prone strains contributes to their IL-12 defect (Fig. 2*B*). Similarly, the addition of a physiologically high concentration (10 ng/ml; see Figs. 1 and 2) of IL-12 to LPS-activated Mϕ had no substantial effect on TNF-α expression, and, therefore, IL-12 is unlikely to be responsible for the defective TNF-α production by MRL/++ and NZB/W F_1 Mϕ (Fig. 3). In fact, IL-12 treatment tended to modestly down-regulate TNF-α production in Mϕ from all strains tested, except A/J, where inhibition of Mϕ IL-12 production was routinely ~50–60% (Fig. 3).

Additionally, IL-12 treatment had no substantial effect on IL-6 production by C57BL/6 or MRL/++ Mϕ, nor did it affect the dramatic ability of TNF-α to correct the progressive MRL/++ Mϕ defect in IL-6 production that occurs after 24 h (Fig. 4). Similar results were obtained with Mϕ from other control strains and from NZB/W F_1 mice (data not shown). IL-12 also had no effect on either normal Mϕ IL-1 expression, or on the defective kinetic production of IL-1 by Mϕ from lupus-prone strains (data not shown). These findings further demonstrate that IL-12 does not substantially modulate expression of these proinflammatory cytokines, and more importantly, that defective IL-12 is not involved in dysregulated expression of the other proinflammatory cytokines by Mϕ from lupus-prone strains. The unresponsiveness of Mϕ to IL-12 is further demonstrated by the observation that TNF-α production by Mϕ from C57BL/6 mice, a strain that produces low levels of TNF-α (15) and substantial levels of IL-12 (see Figs. 1 and 2), is not enhanced by the Ab-mediated neutralization of endogenous IL-12 production (453 ± 50 U/ml with LPS alone and 457 ± 44 U/ml with LPS plus 3 μg/ml anti-IL-12 mAb; IgG1 isotype control was similarly without effect on TNF-α expression).

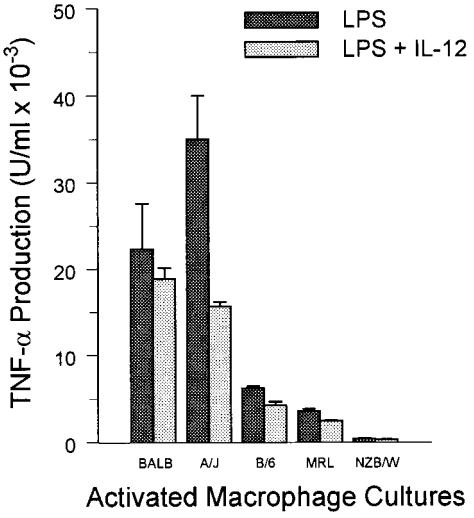

FIGURE 3. IL-12 does not substantially regulate Mϕ TNF-α production. Thioglycollate-elicited peritoneal Mϕ (10^5) from control (BALB/c, A/J, and C57BL/6) and lupus-prone (MRL/++ and NZB/W F_1) murine strains were activated with 100 ng/ml LPS in the absence or presence of IL-12 (10 ng/ml), and conditioned medium was removed at 16 h and assessed for TNF-α levels. Each bar represents the mean and SE of triplicate values. Results are representative of three experiments that had similar results.

Thus, defective expression of IL-1 and IL-6 (15), but not IL-12, arises as a consequence of the early defect in TNF-α expression, and, moreover, defective IL-12 expression does not contribute to defective TNF-α, IL-1, and IL-6 expression.

Lupus-prone Mϕ IL-12 production is stimulated by the Th1-derived cytokine, IFN-γ, in a normal fashion

The compromised ability of lupus-prone Mϕ to produce sufficient levels of IL-12 induced with mitogen alone suggests that these Mϕ would produce suboptimal levels of IL-12 during the initiation of a Th1 response. IL-12 is required for the initial production of the Th1-derived cytokine, IFN-γ, which in turn amplifies the development of Th1-responsive cells by up-regulating Mϕ IL-12 production; therefore, we determined whether lupus-prone Mϕ were appropriately responsive to IFN-γ in their IL-12 production, and thus, would be capable of promoting an ongoing Th1 response. To determine the degree of responsiveness to IFN-γ by Mϕ from lupus-prone strains, Mϕ from control and lupus-prone mice were activated with LPS in the presence or absence of IFN-γ, and conditioned medium was then assessed for IL-12 (Fig. 5*A*). The greatest augmentation (roughly 20-fold) of IL-12 production by IFN-γ treatment occurred in Mϕ from the two lupus-prone strains and the normal BALB/c strain. Strikingly, IFN-γ up-regulated IL-12 production by LPS-activated Mϕ from all control strains in a strictly-defined manner that was inversely proportional to IL-12 levels induced by LPS alone (Fig. 5*B*). Additionally, this response is not simply due to a shift in kinetics because Mϕ from lupus-prone mice continued to demonstrate an enhanced responsiveness to IFN-γ during the intermediate and late intervals, and in response to other doses (0.1 or 10 ng/ml) of IFN-γ as well (data not shown). Interestingly, the low levels of IL-12 produced by the BALB/c strain were restored to normal levels by IFN-γ treatment, but unlike those of lupus-prone strains, were enhanced to precisely the extent predicted by the relative differences among the other control strains (Fig. 5*B*). A likely interpretation of these data is that IFN-γ-signaling is intact in both lupus-prone and BALB/c mice, but the BALB/c strain, although a low IL-12 producer upon stimulation with LPS alone, defines the lower limit of the normal cytokine production spectrum because IL-12 levels were efficiently restored by IFN-γ to those of all other normal strains. However, lupus-prone strains display a truly "defective" pattern of IL-12 expression stimulated with LPS alone because their response to IFN-γ, although intact and operating maximally, did not restore IL-12 levels to those of normal strains. Nevertheless, the dramatic response to IFN-γ by lupus-prone (and by BALB/c) Mϕ, which led to IL-12 levels that approached those of normal strains, suggests that these Mϕ would be capable of propagating, but not initiating, a Th1 response.

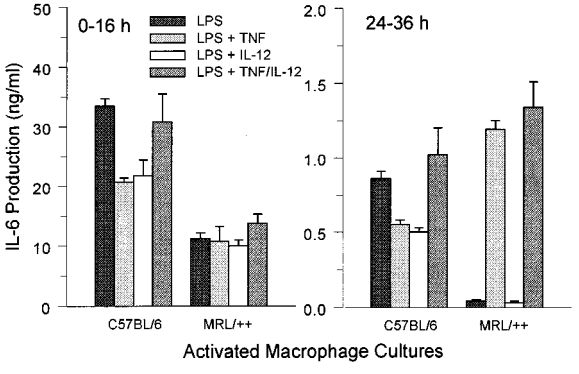

FIGURE 4. IL-12 does not substantially regulate Mϕ IL-6 production. Thioglycollate-elicited peritoneal Mϕ (10^5) from control (C57BL/6) and lupus-prone (MRL/++) mice were activated with 100 ng/ml LPS in the absence or presence of IL-12 (10 ng/ml). Cultures were incubated for three sequential intervals in which conditioned medium was removed and replenished with fresh medium and stimuli for assessment of IL-6. Only the first (0–16 h) and third (24–36 h) intervals are shown for the sake of brevity. Each bar represents the mean and SE of triplicate values. Results are representative of three experiments that had similar results.

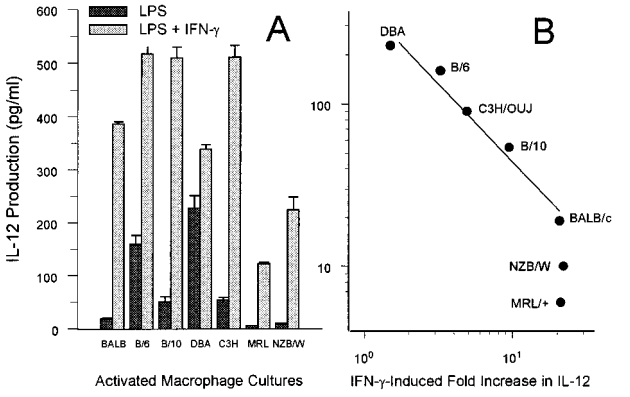

FIGURE 5. IFN-γ reduces the extent of defective IL-12 production in Mϕ from lupus-prone mice. Thioglycollate-elicited peritoneal Mϕ (10^5) from control (BALB/c, C57BL/6, C57BL/10, DBA/2, C3H/OuJ,) and lupus-prone (MRL/++ and NZB/W F_1) murine strains were activated with 100 ng/ml LPS in the absence or presence of IFN-γ (1 ng/ml), and conditioned medium was collected at 16 h and assessed for IL-12 levels. Each bar represents the mean and SE of triplicate values (*A*). The change in IL-12 production in response to IFN-γ treatment was plotted against the IL-12 levels stimulated with LPS alone (*B*). Linear regression analysis for normal strain values in *B* showed that $R^2 = 0.99$. These data represent one of four experiments that had similar results. No IL-12 was detected in Mϕ cultures in the absence of LPS or with IFN-γ alone.

Response to IFN-γ distinguishes the regulation of IL-1, IL-6, and IL-10 production from that of IL-12 and TNF-α

The IL-12 and TNF-α defects may arise from common mechanisms inasmuch as they show similar kinetics (i.e., early expression, see Figs. 1–3, and Ref. 15), expression of one cytokine does

Table I. *Effects of IFN-γ treatment on early cytokine production by Mϕ from normal and lupus-prone murine strains*[a]

Mϕ Strain	IFN-γ	Cytokine Production		
		IL-1 (U/ml × 10^{-3})	IL-6 (ng/ml)	IL-10 (U/ml × 10^{-3})
BALB/c	−	127 ± 15	827 ± 9	90 ± 10
	+	56 ± 2[b]	818 ± 19	1 ± 0[b]
A/J	−	199 ± 35	712 ± 114	50 ± 1
	+	19 ± 0.7[b]	526 ± 11[b]	10 ± 2[b]
C57B1/6	−	22 ± 2	318 ± 7	190 ± 10
	+	5 ± 0.2[b]	350 ± 14	21 ± 3[b]
MRL/++	−	30 ± 3	286 ± 36	30 ± 1
	+	21 ± 3	174 ± 16[b]	0[b]
NZB/WF_1	−	24 ± 5	576 ± 2	435 ± 16
	+	1 ± 0.3[b]	570 ± 2	190 ± 8[b]

[a] Peritoneal Mϕ (10^5) from each murine strain were cultured with LPS (100 ng/ml) in the absence or presence of IFN-γ for 16 h, and cell lysates were analyzed for IL-1 bioactivity using the D10.G4 bioassay and conditioned medium analyzed for IL-6 and IL-10 by ELISA.

[b] Significantly ($p < 0.01$) different from values of cytokine produced in the absence of IFN-γ.

not appear to modulate expression of the other, and the extent of their defects is dramatically reduced by IFN-γ-treatment (see Ref. 15 for IFN-γ regulation of defective TNF-α expression). Conversely, the diminished autocrine stimulation attributable to early defective TNF-α expression (15), and not, as shown here, to the early defective IL-12 production (see Figs. 3 and 4), appears responsible for the downstream IL-1 and IL-6 expression defects in MRL/++ and NZB/W F_1 Mϕ. Thus, while at least part of the mechanism leading to the downstream IL-1 and IL-6 defects has been revealed, the underlying aberrant events responsible for defective TNF-α and IL-12 expression remained to be determined. Moreover, the regulation of these cytokines by IFN-γ also distinguishes two classes of cytokine defects (Table I). Production of TNF-α and IL-12 is strongly stimulated by IFN-γ (see Fig. 5 and Ref. 15, respectively), whereas IL-1 and IL-6 production are either suppressed or not substantially modulated, respectively, by IFN-γ treatment (Table I). Other doses of IFN-γ (i.e., 0.1 and 10 ng/ml) demonstrated similar modulation of IL-1 and IL-6 production in a dose-dependent fashion (data not shown). These data demonstrate that the two classes of cytokine defects, 1) the early onset defects of TNF-α and IL-12, and 2) the later onset, or progressive, defects of IL-1 and IL-6, can be further distinguished by their initial response to IFN-γ in that the earliest aberrant functional events in Mϕ from lupus-prone strains appear to compromise the expression of cytokines that can be strongly stimulated by IFN-γ. We next tested, as a corollary to this finding, whether cytokines that are not dysregulated in lupus-prone Mϕ might also be refractory to IFN-γ-mediated stimulation. IFN-γ treatment did not stimulate and, in fact, suppressed IL-10 production by LPS-stimulated Mϕ from control and lupus-prone strains (Table I), supporting the contention that only cytokines stimulated during early periods by IFN-γ (i.e., IL-12 and TNF-α) show early defective expression.

Discussion

Determination of genetically programmed (i.e., intrinsic) functional defects is critical to our understanding the origins of autoimmunity. One approach lies in genetic analysis, and such studies have identified linkage groups that confer sensitivity to disease (22–24). Another approach is to identify defects in immune cell function that appear early in development, before the onset of any disease signs, and thus have the potential to initiate as well as propagate disease. In the latter category, it has been difficult to demonstrate substantial lymphocyte-associated intrinsic defects that are shared among the lupus-prone strains, such as MRL/++ and NZB/W F_1, whose disease is multigenic in origin (25, 26). However, we have reported that these strains are characterized by conserved defects in Mϕ production of at least three proinflammatory cytokines, TNF-α, IL-1, and IL-6, that may be fundamental to the disease process (9, 10, 15). In addition, Mϕ from both strains are also characterized by normal IL-10 expression, and by the hierarchy of TNF-α that, by virtue of its under-expression, causes the subsequent defects in IL-1 and IL-6 (15). That the defects are readily apparent in Mϕ obtained from young, prediseased mice, are common to both lupus-prone strains, and are intrinsic in nature, suggest that aberrant expression of these immunomodulatory cytokines may play a critical role in the initiation and development of the disease process. Indeed, the critical contribution of these defects in Mϕ-derived cytokines has been demonstrated by the therapeutic effect of administration of TNF-α or IL-1 to young, lupus-prone, NZB/W F_1 mice before the onset of disease (12, 27, 28). Here, we demonstrate a novel and dramatic defect in MRL/++ and NZB/W F_1 Mϕ production of IL-12, a cytokine that is critical in the development of Th1 responses (16). This IL-12 defect was manifested in a manner similar to that of TNF-α (15) in that both defects developed rapidly after activation with LPS and could be substantially normalized by treatment with exogenous IFN-γ. However, defective expression of IL-12, unlike that of TNF-α (15), did not contribute to the subsequent IL-1 and IL-6 defects in Mϕ from lupus-prone mice because addition of rMuIL-12 (or the addition of anti-IL-12 mAb) did not substantially regulate IL-1 or IL-6 production. Furthermore, the IL-12 and TNF-α defects did not contribute to each other because addition of either cytokine had no substantial regulation of expression of the other. These findings strengthen the premise that cytokine dysregulation defines a conserved and perhaps dominant pathway leading to systemic autoimmunity in those strains whose disease, like human lupus, is multigenic in origin.

Earlier we had reported evidence for two distinct classes of cytokine defects expressed in both MRL/++ and NZB/W F_1 Mϕ (15). These are defined operationally by their time of expression after Mϕ stimulation: expression of the TNF-α defect preceded expression of the defects in IL-1 and IL-6, and, moreover, caused the latter defects. The IL-12 defect reported here falls into the former category based on kinetics, yet is distinct from TNF-α in that it neither precipitated nor modulated the ensuing defects in IL-1 and IL-6. The two classes of cytokine defects defined above can be further distinguished by their responsiveness to IFN-γ, an NK and T cell-derived cytokine known to enhance Mϕ cytokine

production (29). IFN-γ treatment dramatically stimulated LPS-induced IL-12 and TNF-α production (15) by Mϕ from all strains, and the response was noted to be inversely proportional to the basal level of cytokine produced upon stimulation with LPS alone. Conversely, IFN-γ suppressed the early (16 h) production of IL-1 and did not substantially regulate IL-6 expression, these targets being representative of the second class of cytokine defects. This selective inhibitory activity of IFN-γ on IL-1 (as well as on IL-10), and its lack of regulation of IL-6 production, have also been reported by others (30, 31). It is reasonable that the cytokine defects of the first class (i.e., TNF-α and IL-12) are caused by one or more underlying aberrant events that can be overcome or normalized by IFN-γ-induced signals that normally stimulate those cytokines. These stimulatory signals arising from the IFN-γ receptor may include both secondary messenger molecules, such as JAK 1 and 2, and STAT1α molecules, and several transcription factors (32) and may provide clues as to the aberrant molecular events that arise in these strains.

Although IFN-γ dramatically suppressed LPS-induced production of the autocrine inhibitory cytokine IL-10, it is unlikely that IFN-γ reduced the extent of defective TNF-α and IL-12 expression in MRL/++ and NZB/W F_1 Mϕ solely by suppressing IL-10 for the following reasons: 1) The autocrine inhibitory activity of IL-10 does not appear to be involved in defective TNF-α (15) or IL-12 production (our unpublished observation) because Ab-mediated neutralization of IL-10 did not reduce the extent of these defects. 2) LPS-activated Mϕ from MRL/++ and NZB/W F_1 strains do not over-express IL-10 (15). 3) The IFN-γ-induced augmentation of TNF-α (15) and IL-12 production among control Mϕ is proportional to their IL-10 levels (15), suggesting that IFN-γ may normalize TNF-α and IL-12 production by inhibiting IL-10; however, clearly the TNF-α and IL-12 response to IFN-γ by Mϕ from lupus-prone mice is not proportional to their IL-10 levels (i.e., MRL/++ have lower IL-10 and NZB/W F_1 have higher IL-10 levels than BALB/c (15)). Therefore, IFN-γ appears to normalize TNF-α and IL-12 expression in MRL/++ and NZB/W F_1 Mϕ by an IL-10-independent mechanism.

These findings suggest the possibility that the aberrant expression of TNF-α and IL-12 could develop as a consequence of an up-stream defect in IFN-γ expression by these LPS-activated Mϕ. There is evidence that LPS-activated murine peritoneal Mϕ express minute levels of IFN-γ mRNA along with intracellular, but not extracellular, expression of translated IFN-γ (33). Although it was suggested that intracellular IFN-γ may cooperate with LPS to induce TNF-α expression (33), and while we have confirmed Mϕ IFN-γ production by RT-PCR and Southern blot analysis (our unpublished observation), we did not find defective expression of LPS-activated IFN-γ mRNA levels in Mϕ from lupus-prone strains by these procedures (our unpublished observation), nor could we detect secreted IFN-γ by ELISA, or modulate TNF-α or IL-12 production with anti-IFN-γ mAb treatment (our unpublished observation). These results suggest that the mechanisms underlying dysregulated TNF-α and IL-12 production do not include dysregulated endogenous IFN-γ production.

Defective expression of IL-12, in cooperation with defective TNF-α and IL-1, by Mϕ from lupus-prone mice may have an impact on disease both during and after initiation of the disease process. The initial polyclonal hyperexpansion of ubiquitous autoreactive lymphocytes that appears as the first stage of disease (1, 2) may be linked to defective TNF-α-induced apoptosis in addition to diminished suppressor activity by IFN-γ, an NK- and T cell-derived cytokine normally stimulated by the combination of TNF-α, IL-1, and IL-12 (34–36), all of which we have found to be defectively produced by MRL/++ and NZB/W F_1 Mϕ (17). Following this polyclonal hyperexpansion of autoreactive lymphocytes, defective IL-12 expression may also permit the development of the strong, Ag-specific Th2-mediated humoral responses, such as those directed toward chromatin (24), that characterize later stages of lupus. Indeed, administration of IL-12 blocks pathogenic autoantibody production and end-stage renal pathology in lupus-like, mercury-induced autoimmune disease in mice (37). Moreover, that IL-12 administration to cultures of PBMC from human SLE patients directly suppresses the excessive spontaneous production of Ig and anti-DNA IgG Ab (38), along with the reduced IL-12 and IFN-γ levels produced by these cultures (39, 40), suggests that defective IL-12 levels in human SLE leads to hypergammaglobulinemia and the generation of pathogenic Ab. Furthermore, it has recently been reported, using a T cell receptor transgenic mouse, that Mϕ-derived TNF-α and IL-1 are required for IL-12 to induce development of a Th1 population from naive precursors in vitro (41). Thus, the defective TNF-α and IL-1 expression by Mϕ from lupus-prone strains, in concert with defective IL-12 production, could play a fundamental role in lupus by fostering the polyclonal hyperexpansion of autoreactive lymphocytes and the subsequent establishment of a Th1-deficient environment, which, ultimately, would lead to enhanced production of pathogenic autoantibodies.

The physiological relevance of these findings will be determined, in part, by whether the extent of defective IL-12 expression noted in the lupus-prone strains is sufficient to alter the balance of T cell subsets or otherwise promote pathogenic Ab responses. In this light, it is noteworthy that the low IL-12 expression by Mϕ from BALB/c mice shown here correlates with the sensitivity of this strain to infection by the parasite, *L. major* (20). BALB/c mice are unable to lodge a protective Th1 response against *L. major* infection, and this is associated with exuberant IL-4 production and a strong Th2 response (20). Conversely, C57BL/6 and C3H/OuJ strains, whose Mϕ produce dramatically more IL-12 than do BALB/c Mϕ, are resistant to infection and mount a strong Th1 response against this parasite. These findings thus suggest a genetic basis for BALB/c susceptibility to *L. major* infection linked to deficient IL-12 production. Indeed, it has been demonstrated, using *L. major*-resistant strains created from congenic backcrosses of BALB/c and C57BL/6 strains, that disease susceptibility of BALB/c mice segregated with a region of chromosome 11 that contains the IL-12 (p40) gene (42). If the low levels of IL-12 produced by BALB/c Mϕ do contribute to enhanced IL-4 expression and *L. major* susceptibility in this strain, then the more dramatically reduced IL-12 levels in the MRL/++ and NZB/W F_1 strains are likely to be of similar, or even greater, physiological significance, and as such, may contribute to autoimmunity. All the cytokine defects that we have reported are found in both MRL/++ and NZB/W F_1 Mϕ, and, moreover, are expressed before any detectable disease signs. Thus, it is tempting to speculate that the two defective pathways discussed earlier, 1) reduced TNF-α, leading to reduced IL-1 and IL-6, and 2) reduced IL-12, may both be important for establishing lupus of multigenic origin.

Our findings demonstrate that defective Mϕ proinflammatory cytokine production has been conserved among at least two genetically distinct backgrounds prone to murine lupus, suggesting a common, intrinsic cellular aberrancy that might be diagnostic for this disease and, more importantly, may be involved in the initiation and propagation of the disease process. Clearly, the contribution of the Mϕ defects described here must be viewed in the context of the gene mapping studies which have revealed multiple genetic linkage groups associated with specific aspects of murine lupus (24). In particular, the elegant genetic studies of Wakeland

and coworkers (22, 24) and Kotzin and coworkers (23) have revealed loci which are associated with B cell hyperactivity. Although there are claims of intrinsic defects in B lymphocytes function from lupus-prone strains, the splenic B cell populations studied generally are the product of T cell depletion, and thus enriched both for B cells and Mϕ (5, 6, 22). Neither percoll enrichment of B cells nor adherence-based elimination of Mϕ are particularly helpful procedures due to the marked ability of spleen cells to generate Mϕ from immature, nonadherent precursors in culture (our unpublished observations). While the use of purified long-term B cell lines transferred into SCID mice provides more compelling support for intrinsic B cell defects (6), these animals do not manifest all aspects of NZB/W F_1 B cell hyperactivity, in keeping with the concept that the complexity of lupus is most likely due to several mutations operating at different times in development or simultaneously in different cells types. Our findings here place the Mϕ, among other cells of the innate immune system, in a central position regarding the initiation and development of autoimmunity. Moreover, they raise questions as to whether defects ascribed to B and T cells in these multigenic models of lupus (MRL/++, NZB/W F_1) are intrinsic to lymphocytes, or alternatively, reflect differences in lymphocyte development or regulation that arise as a consequence of aberrant control by accessory cells. It is anticipated that continued efforts to understand the control and function of cells regulating innate immunity will provide insight into the ways that dysregulated cytokine expression can contribute, not only as a possible effector mechanism of the end-stage inflammation of the disease process, but also to the fundamental immune dysregulation that initiates the development of lupus.

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