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Aberrant Production of IL-12 by Macrophages from Several Autoimmune-Prone Mouse Strains Is Characterized by Intrinsic and Unique Patterns of NF-κB Expression and Binding to the IL-12 p40 Promoter[1]

Jiajian Liu and David Beller[2]

Intrinsic defects in macrophage (Mϕ) cytokine production characterize many autoimmune-prone mouse strains. Aberrant levels of IL-12, for example, are produced by Mϕ isolated from young mice prone to lupus (MRL and NZB/W) and diabetes (nonobese diabetic (NOD)) well before the appearance of disease signs. Evaluation of the possible mechanism(s) underlying the abnormal regulation of IL-12 in these strains revealed novel patterns of Rel family protein binding to the unique p40 NF-κB site in the IL-12 p40 promoter, whereas binding patterns to Ets and CCAAT enhancer binding protein/β sites were normal. In particular, the heightened production of IL-12 by NOD Mϕ is associated with elevated levels of the *trans*-activating p50/c-Rel (p65) complex compared with the nonfunctional p50/p50 dimer. Conversely, the dramatically impaired production of IL-12 by both NZB/W and MRL/+ Mϕ is associated with a predominance of p50/p50 and reduced p50/c-Rel(p65) binding. Mechanistically, the unique pattern seen in the lupus strains reflects elevated p50 and reduced c-Rel nuclear protein levels. In NOD extracts, the level of c-Rel is elevated compared with that in lupus strains, but not when compared with that in normal A/J. However, the extent of c-Rel tyrosine phosphorylation noted in NOD extracts is more than double that seen in any other strain. Levels of p65 were similar in all strains tested. These findings reveal that a common mechanism, involving dysregulation of c-Rel and p50, may be used to determine the aberrant IL-12 levels that have the potential to predispose specific mouse strains to systemic or organ-specific autoimmunity. *The Journal of Immunology*, 2002, 169: 581–586.

Cytokines are critical for the regulation of both normal physiology and the pathology of autoimmune disease (1, 2). Macrophages (Mϕ)[3] from several autoimmune-prone mouse strains are characterized by intrinsic defects in cytokine production (3–9). These defects can be elicited ex vivo before the onset of disease signs, and the intrinsic nature of some of the cytokine defects has been established (10). Of particular note, Mϕ from the NZB/W and MRL/+ lupus-prone strains produce exceedingly low levels of IL-12, whereas Mϕ from the nonobese diabetic (NOD) and SJL strains prone to organ-specific autoimmunity produce uniquely high levels. In the latter group, the defects in IL-12 correlate with levels of expression of the p40, rather than the p35, subunit of the IL-12 heterodimer (7, 8).

Such intrinsic defects in the regulation of IL-12 may prove particularly valuable in understanding the development of autoimmunity. IL-12 plays a critical role in the selection and maintenance of the Th1 subset (1), which, in turn, is required for eliciting organ-specific autoimmunity (11). Moreover, IL-12 has been shown to inhibit B cell function (12, 13), suggesting that the reduced production of this cytokine in the MRL/+ and NZB/W strains could contribute to lupus as well. In the current study we have addressed the possible mechanisms underlying IL-12 dysregulation. The evaluation of Mϕ NF binding by EMSA revealed that in all strains tested the p40 κB site binds p50 and c-Rel, with lesser involvement of p65. Nuclear extracts from normal Mϕ express ostensibly similar levels of DNA-binding p50/c-Rel(p65) and p50/p50 complexes. In contrast, in NOD Mϕ, binding of the p50/c-Rel(p65) *trans*-activating complex is clearly dominant and reflects an increased fraction and total amount of tyrosine-phosphorylated (p-Tyr) c-Rel. Strikingly, this pattern is the reverse of that seen in MRL/+ and NZB/W Mϕ, which both show a marked elevation in binding of the inhibitory p50 homodimer. The latter pattern accurately reflects nuclear levels of c-Rel and p50 proteins in Mϕ from these strains. Comparison of Mϕ from six strains revealed a rigorous correlation between the pattern of κB binding and IL-12 production. These findings suggest that a common mechanism, perturbed κB/Rel binding, may be used to direct the aberrant IL-12 levels that have the potential to predispose individuals to systemic or organ-specific autoimmunity.

Materials and Methods

Mice

Five-week-old male mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed on-site for an additional week before use.

Mϕ isolation and culture

Mϕ were obtained from peritoneal exudate cells (PEC) by peritoneal lavage with cold RPMI 1640 medium supplemented with 5% FBS, 1% L-glutamine, 0.5% HEPES, and 1% penicillin/streptomycin (BioWhittaker,

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[3] Abbreviations used in this paper: Mϕ, macrophage; c, consensus (sequence); mIL-12, mouse IL-12; PEC, peritoneal exudate cell; p-Tyr c-Rel, tyrosine-phosphorylated c-Rel; RPA, RNase protection assay; C/EBPβ, CCAAT enhancer binding protein β.

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Walkersville, MD) 4 days after i.p. injection of thioglycolate (2 ml; Remel, Lenexa, KS). Mϕ were seeded either at 1.5×10^5 cells in 100 μl medium/well of 96-well plates for cytokine production (assessed by ELISA) or at 10^7 cells in 10 ml medium/100-mm-diameter dish for cytokine RNA measurements (using RNase protection assay (RPA)) or NF (assessed by EMSA). PEC were allowed to adhere 3 h at 37°C in 5% CO_2. Nonadherent cells were removed by washing in medium to provide cultures routinely comprising ≥95% adherent Mϕ. Mϕ were activated with 100 ng/ml LPS, as indicated below.

ELISA

Mϕ were activated with LPS for 16 h, and conditioned medium was collected to assess IL-12. IL-12 heterodimer (p70) and IL-12 p40 subunit levels were quantitated by OptEIA mouse IL-12 (mIL-12) p70 (catalog no. 2661KI) and mIL-12 p40 (catalog no. 2619KI) ELISA kits (BD PharMingen, San Diego, CA), following the manufacturer's instructions.

RNase protection assay

Mϕ were activated with LPS for 0, 4, or 8 h, at which time total RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD). Ten micrograms of total RNA was assessed using a multiprobe RPA kit with the cytokine/chemokine template set mCK-2b (BD PharMingen), following the manufacturer's directions (catalog no. 556850).

EMSA

Nuclear extracts were prepared as described previously (14). Sequences of EMSA oligonucleotide probes are displayed in the legends of Figs. 2 and 3. Probes were made by annealing single-strand oligonucleotides (Life Technologies) with 5′-GATC overhangs and were labeled by filling in with [α-^{32}P]dATP (NEN Life Science Products, Boston, MA) using Klenow enzyme. The binding reaction mixture contained 10,000 cpm labeled probe, 3 μg nuclear protein, 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 2 μg poly(dI-dC) (Amersham Pharmacia Biotech, Piscataway, NJ), and 10% glycerol in a final volume of 20 μl. The mixture was incubated for 30 min at room temperature, and complexes were separated by PAGE (nondenaturing gel, 6% in 1× Tris-glycine-EDTA, pH 8.3) at 4°C for 1.5 h at 300 V. The gel was dried and visualized by autoradiography. For the competition analyses, a 100-fold molar excess of competitors was incubated with the nuclear extracts (20 min at room temperature) before addition of the labeled probe. For the supershift assays 2 μg of the appropriate Abs (Santa Cruz Biotechnology, Santa Cruz, CA; see below) were added to the reaction mixtures (30 min at room temperature) before addition of the labeled probe.

Immunoblot assay

Nuclear extracts (16 μg) were fractionated on 7.5% SDS-polyacrylamide gels. The separated proteins were transferred to a Trans-Blot transfer membrane (catalog no. 162-0145, Bio-Rad, Hercules, CA), which was then probed by an anti-actin polyclonal Ab (catalog no. sc-1615). HRP-conjugated secondary Abs (catalog no. sc-2352 and sc-2054) were detected using chemiluminescence luminol reagent (catalog no. sc-2048). The membrane was stripped and probed, in turn, by c-Rel, p50, and p65 polyclonal Abs (catalog no. sc-71x, sc-114x, and sc-372x, respectively). Anti-phosphotyrosine Ab (catalog no. 05–321) was purchased from Upstate Biotechnology (Lake Placid, NY) and all Abs and reagents were purchased from Santa Cruz Biotechnology, except as indicated. For immunoblotting after immunoprecipitation, 16 μg of nuclear extracts were immunoprecipitated with the c-Rel Ab, and then fractionated by PAGE. Proteins thus separated were immunoblotted with the anti-phosphotyrosine Ab. To control for loading, the same membrane was then stripped and probed with the c-Rel Ab.

Results

IL-12 p40 mRNA levels are elevated in NOD and reduced in NZB/W and MRL/+ Mϕ

We had shown earlier that both NZB/W and MRL/+ Mϕ show a striking deficiency of IL-12 p40 and p70 production compared with Mϕ from the normal A/J strain (7), and that NOD Mϕ produce substantially higher levels than normal (9). These studies had compared the autoimmune-prone strains to an extensive panel of normal strains, verifying both the relatively consistent behavior of Mϕ from the normal strains and the polarized behavior of Mϕ from the lupus strains compared with NOD. We had established that for NOD Mϕ, heightened IL-12 levels were associated with correspondingly elevated p40, but not p35, mRNA (9). The data shown in Fig. 1, *A* and *B*, confirm this observation and demonstrate the novel finding that the reduced IL-12 levels noted in both NZB/W and MRL/+ Mϕ (7) (see also Fig. 4A) are associated with correspondingly reduced p40 mRNA.

Unique NF-κB binding patterns distinguish A/J, NOD, and NZB/W Mϕ

Based on these results, nuclear extracts from LPS-activated Mϕ were tested by EMSA for binding to sites known to be important in regulation of IL-12 p40 transcription (15–17). The goal was to determine whether specific patterns of NF binding might correlate with the dysregulated expression of p40 mRNA seen in the Mϕ from autoimmune-prone strains. The data in Fig. 2*A* reveal unique patterns of κB binding in extracts prepared 2 h after Mϕ activation. The supershift data in Fig. 2, *B* (A/J), *C* (NOD), and *D* (NZB/W), reveal that binding to the κB site in the p40 promoter is composed predominantly of p50 and c-Rel. Lesser amounts of p65 also bind to this site, so we have referred to this complex as p50/c-Rel (p65). The efficacy of the p65 Ab was demonstrated by its successful supershifting of T cell-derived nuclear proteins bound to the Fas ligand promoter κB site (data not presented). A/J Mϕ (Fig. 2) display ostensibly similar levels of p50/p50 and p50/c-Rel(p65) binding to the p40 κB sequence. Strikingly, NF from Mϕ of the autoimmune-prone strains differ from normal Mϕ NF in a highly reproducible and functionally relevant manner. NOD Mϕ, which display elevated p40 production, have a binding profile favoring the functional heterodimer. Conversely, NZB/W Mϕ, with reduced p40 production, have an abundance of the inhibitory p50 homodimer. Thus, it is the unique ratio of κB complexes, rather than generic hypo- or hyperexpression of NF-κB or use of distinct Rel proteins, that appears to distinguish Mϕ from the different disease-prone strains. Similar results were noted at 0.5 and 1 h (data not presented), suggesting that these unique patterns are intrinsic to the Mϕ and do not result from autocrine regulation, and that they are stable rather than transient.

To assess the specificity of this expression pattern, other NF known to regulate p40 transcription (16, 17) were also evaluated. Because these NF (CCAAT enhancer binding protein β (C/EBPβ) and Ets) are late acting (16, 17), they were routinely assessed at the time of optimal expression (8 and 16 h). The results reveal binding

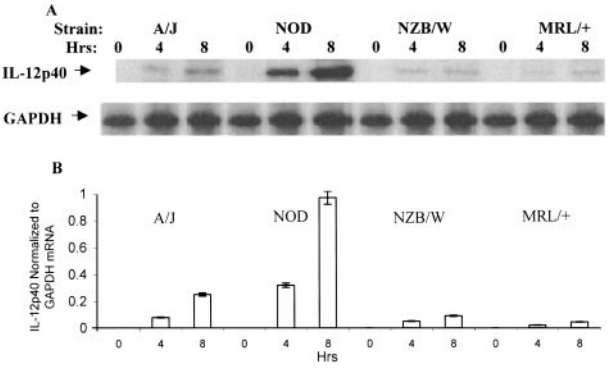

FIGURE 1. Mouse IL-12 p40 mRNA levels are elevated in NOD and reduced in NZB/W and MRL/+ Mϕ. *A*, Thioglycolate-elicited peritoneal Mϕ from the indicated strains were activated with 100 ng/ml LPS for 0, 4, and 8 h, at which time total RNA was extracted, and mIL-12p40 mRNA levels were assessed by RPA. The data represent one of three independent experiments with similar results. *B*, The level of p40 mRNA was quantified by densitometry and normalized to GAPDH controls. Each bar represents the mean and SE of triplicate values.

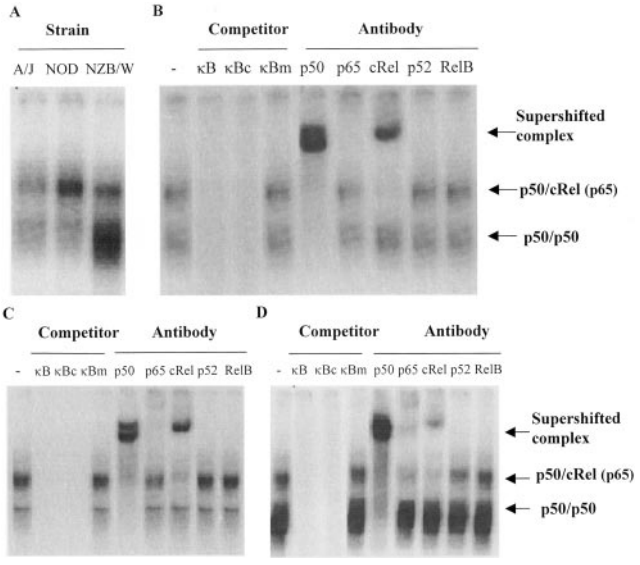

FIGURE 2. Distinct patterns of Rel protein binding to the NF-κB site of the mIL-12 p40 promoter in normal and autoimmune-prone strains. Nuclear extracts were prepared from thioglycolate-elicited peritoneal Mϕ that had been activated with 100 ng/ml LPS for 2 h and were tested for DNA binding by EMSA. Data are representative of four experiments that showed similar results. *A*, Nuclear extracts (3 μg) from A/J, NOD, and NZB/W Mϕ show distinct binding patterns to the p40 NF-κB oligonucleotide (sequence, 5′-CTT CTT AAA ATT CCC CCA GA-3′; location, −137 to −118; the underlined portion is the region of NF-κB homology within the sequence) (15). *B*, Nuclear extracts from A/J mice Mϕ were examined by competition and supershift EMSA, as described in *Materials and Methods.* Lanes (from *left* to *right*) represent nuclear extract and probe incubated with: −, neither competitor nor Ab; κB, excess unlabeled p40 NF-κB oligonucleotide; κBc, excess unlabeled NF-κB oligonucleotide (sequence, 5′-TTGA GGG GAC TTT CCC AGGC-3′; the underlined portion is the NF-κB consensus site); and κBm, excess unlabeled p40 NF-κB 3′ mutation (sequence, 5′-CTT CTT AAA AGG TAT CCA GA-3′; the underlined portion is the mutated region in the κB site). Supershifting was performed with the indicated anti-Rel polyclonal Abs. *C* and *D*, Competition and supershift EMSA for NOD and NZB/W Mϕ extracts, respectively. Competitors and Abs are the same as those in *B*.

of Mϕ NF to the C/EBPβ (Fig. 3*A*) and Ets (Fig. 3*B*) consensus sequences that is indistinguishable among the three strains. The pattern of binding to both probes was also similar among the strains at earlier time points (1 and 2 h), although the level of binding was low (data not presented). Thus, to the extent examined, the unique NF binding patterns that distinguish A/J, NOD, and NZB/W Mϕ appear to be specific for the NF-κB site.

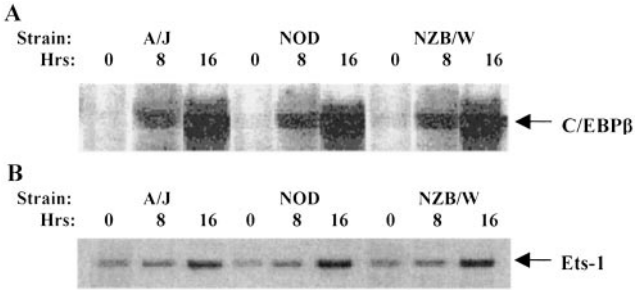

FIGURE 3. NF binding to C/EBPβ and Ets sequences in the p40 promoter is consistent among strains. *A* and *B*, Nuclear extracts were evaluated for binding to other regulatory sites in the p40 promoter. Extracts were prepared as described in Fig. 2, except that extracts were prepared 8 and 16 h after activation. Data represent one of three independent experiments that showed similar results. The location in p40 promoter and sequence of the oligonucleotides used is: *A*, −101 to −83 (5′-TTT CA GTG TTG CAA TTG AG-3′; the underlined portion is the C/EBPβ site) (15); and B, −224 to −207 (5′-AAG TCA TTT CCT CTT AAC-3′; the underlined portion is the Ets site) (15, 16).

Balanced homo- and heterodimer binding activity characterizes Mϕ from normal, but not autoimmune-prone, strains

We next compared Mϕ from several normal strains to determine whether they display a consistent pattern of NF binding to the p40 κB site, characterized by ostensibly similar levels of p50/c-Rel(p65) and p50/p50. We also evaluated MRL/+ Mϕ to determine whether there was evidence for a consensus pattern among lupus-prone strains. As shown in Fig. 4*A*, each of the three normal strains tested (A/J, B/6, and BALB/c) produced very similar levels of IL-12 p40 and p70 proteins. This is in striking contrast to production of the cytokines TNF and IL-10, which varies by over 20-fold among these strains (6). Thus, regulation of IL-12 production is unusual in that it is normally held within rigid limits. Clearly, Mϕ from autoimmune-prone strains are aberrant in IL-12 production: NOD Mϕ produce heightened p70 and p40 levels (410 and 350% of the mean normal values, respectively), whereas Mϕ from both lupus strains produced low levels (NZB/W, 24 and 49%; MRL/+, 11 and 25% of the mean normal p70 and p40 levels, respectively).

Evaluation by EMSA (Fig. 4*B*) demonstrated similar binding of the two dominant Rel complexes in Mϕ NF obtained from each normal strain, and these findings were quantitated by densitometry in Fig. 4*C*. Thus, this pattern is consistent with the conserved levels of IL-12 production seen in normal strains (Fig. 4*A*) (7–9). Additionally, the data reveal that the IL-12-deficient Mϕ from the MRL/+ strain, which displays late-onset lupus independent of the *lpr* mutation, share the same κB perturbation (dominance of p50/p50 binding) seen in NZB/W Mϕ. Taken together, these "experiments of nature" suggest that divergence from the normal balance of κB binding leads to the aberrant IL-12 levels that characterize each of the autoimmune-prone strains tested. This interpretation is supported by the regression analysis seen in Fig. 4*D*; there is a virtually absolute correlation of the κB binding pattern (ratio of hetero- to homodimer) and the amount of p40 and p70 proteins produced.

Additional findings (our unpublished observations) further support this view. First, Mϕ from the NOD-congenic, but diabetes-resistant, NOR strain, which produce normal levels of IL-12 (9), express the normal balance of hetero- and homodimer. This reveals that in the NOR, a strain of near genetic identity to the NOD, there is conserved 1) genetic resistance to disease, 2) normal Mϕ IL-12 production, and 3) normal Rel family binding to the p40 κB site. Second, we have found that the defect in NZB/W Mϕ IL-12 production is derived from the NZW, not the NZB, parent (D. Alleva and D. Beller, unpublished observations). Preliminary findings indicate that the prevalence of p50 homodimer binding seen the NZB/W is likewise associated with the NZW parent. Taken together, these findings suggest a common mechanism underlying the bias toward dysregulated Mϕ IL-12 production characteristic of autoimmune-prone mice.

Levels of nuclear p50 and c-Rel in NZB/W and MRL/+, but not in NOD, reflect their DNA binding activity

In an effort to determine the basis for the unique patterns of κB binding in the different strains, nuclear extracts were fractionated by SDS-PAGE, and proteins were identified by immunoblotting (Fig. 5*A*). The results reveal that for both lupus strains (NZB/W and MRL/+), nuclear c-Rel levels are reduced, while p50 is increased, relative to those in A/J mice. The pattern in NOD, by contrast, appears similar to that in A/J. The relative levels of p50,

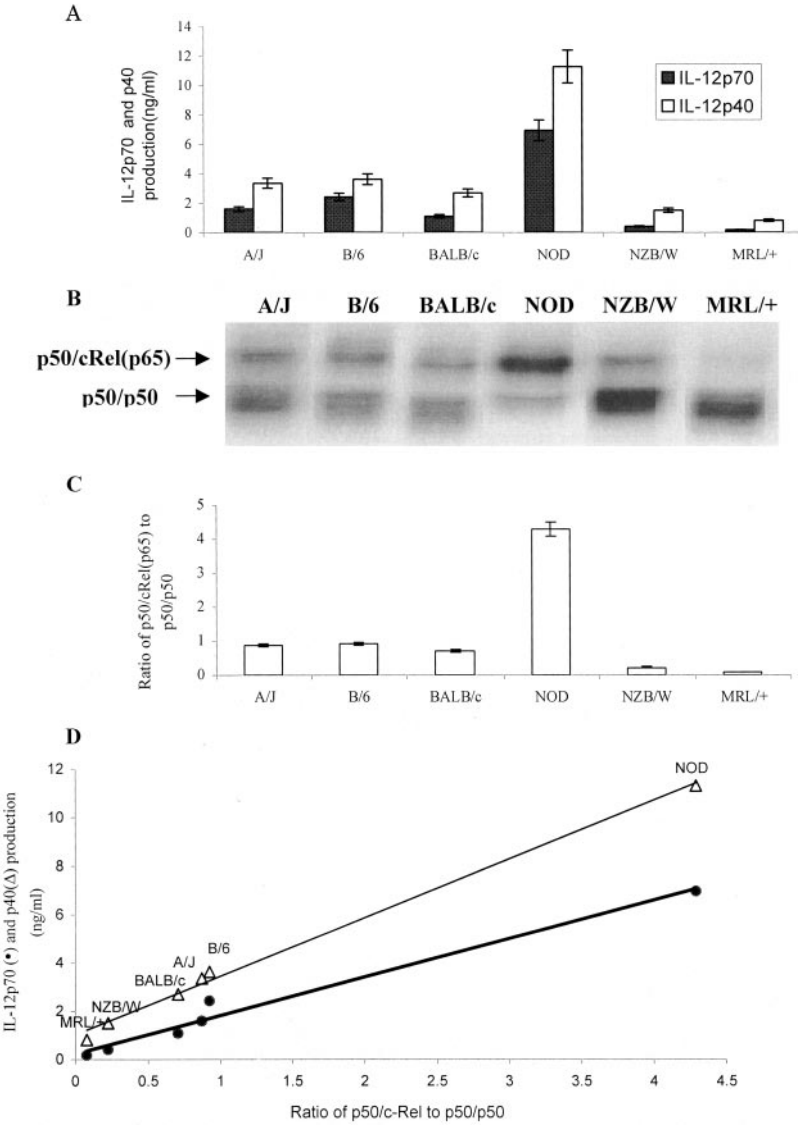

FIGURE 4. Mϕ extracts from normal and autoimmune-prone mice display unique patterns of NF-κB binding that correlate with IL-12 production. *A*, IL-12 p70 and p40 proteins are elevated in NOD Mϕ and reduced in NZB/W and MRL/+ Mϕ compared with those in Mϕ of several normal strains. Thioglycolate-elicited peritoneal Mϕ were activated with 100 ng/ml LPS for 16 h. Conditioned media were assessed by ELISA for both IL-12p70 and p40 levels. Each bar represents the mean and SE of triplicate values. *B*, A unique pattern of NF-κB binding to the p40 promoter in seen in Mϕ extracts from normal and autoimmune-prone mice. Specifically, the ratio of p50/c-Rel(p65) to p50/p50 is substantially elevated in NOD and reduced in NZB/W and MRL/+ compared with Mϕ from normal strains (*C*). The ratios were quantified by densitometry, using average density per band. A virtually identical profile was obtained using the total density per band (not shown). Each bar represents the mean and SE of triplicate values. *D*, Linear regression analysis comparing IL-12 p70 and p40 levels (from *A*) to the ratio of p50/c-Rel to p50/p50 (from *C*) in normal and autoimmune-prone mice. $R^2 = 0.98$ for IL-12 p70, and 0.995 for IL-12 p40. The strain designations appearing over the upper symbols (p40) also apply to the corresponding p70 values directly below.

c-Rel, and p65 were then determined by densitometry, adjusted for actin levels in each extract, and normalized to the adjusted A/J Rel level, arbitrarily set at 1 (Fig. 5*B*). The relative levels of p65 served as an internal control, being nearly identical in all strains; p65 expression normalized to A/J was 0.99, 0.91, and 1.05 for NZB/W, MRL/+, and NOD, respectively. The extent of reduction in the ratio of c-Rel to p50 protein in Mϕ nuclear extracts from the lupus-prone strains compared with that in normal mice is consistent with the reduction in each of the following: 1) p50/c-Rel(p65):p50/p50 binding to the p40 κB site (EMSA), 2) the level of p40 mRNA (RPA), and 3) p40 and p70 protein levels (ELISA). Thus, the ratio of c-Rel to p50 may be sufficient to explain the reduced activity of the p40 promoter in these two lupus-prone strains. This is not the case in extracts from NOD Mϕ, where both the relative level of nuclear Rel proteins and the ratio of c-Rel to p50 are indistinguishable from those in A/J. This suggests that in the NOD Mϕ a factor other than the level of nuclear Rel protein determines the specific pattern of Rel binding to the κB site.

The level of c-Rel binding in NOD nuclear extracts correlates with the level of c-Rel tyrosine phosphorylation

Phosphorylation is one means by which the function of c-Rel is post-translationally regulated (18). To test the possibility that phosphorylation of nuclear c-Rel is elevated in NOD Mϕ, nuclear proteins were immunoprecipitated with anti-c-Rel Ab, separated by PAGE, and then immunoblotted with anti-phospho-tyr Ab. The same membrane was stripped and immunoblotted with c-Rel to determine relative c-Rel levels. As shown in Fig. 6*A*, A/J and NOD nuclear extracts contained similar levels of c-Rel, which were higher than c-Rel expression in lupus strains. However, tyr-phosphorylated c-Rel (p-Tyr c-Rel) was elevated in the NOD, both in absolute level and as a fraction of the total c-Rel. The results were then quantitated by densitometry (Fig. 6*B*) and normalized to the levels found in A/J (each was arbitrarily assigned a value of 1 for A/J) to more accurately determine the relative expression among strains. The fraction of c-Rel that is tyr-phosphorylated is ~2.5 times greater in NOD than in normal or lupus strains. The amount of p-Tyr c-Rel in NOD was nearly 3 times that in A/J, 6 times that in NZB/W, and 12 times the level in MRL (based on equivalent protein loading). Thus, phosphorylation of c-Rel may be critical in determining the unique κB binding patterns seen in NOD Mϕ.

Discussion

In this report we have linked specific IL-12 defects with unique patterns of Rel binding that are consistent with both the nature of specific autoimmune diseases and the known biological functions

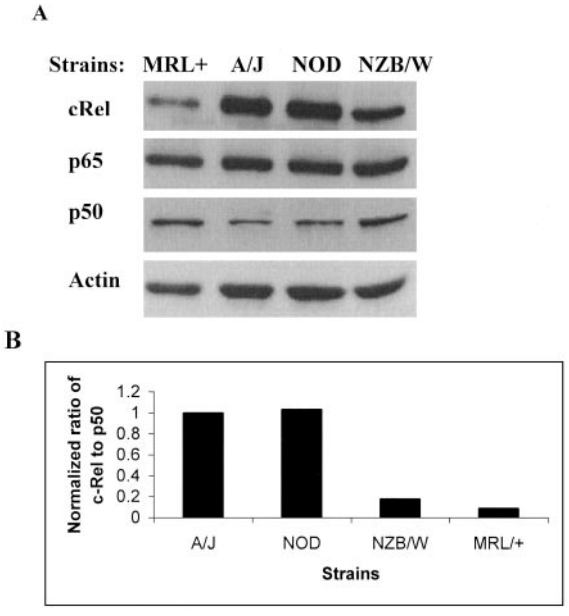

FIGURE 5. Nuclear Rel protein levels in both lupus strains, but not in NOD, are consistent with their DNA binding activity. *A*, Rel proteins in nuclear extracts from A/J, NOD, NZB/W, and MRL/+ PEC were detected by immunoblotting using c-Rel, p65, p50, and actin Abs. *B*, Levels of Rel proteins were measured by densitometry and normalized to actin. The ratio of c-Rel to p50 in each autoimmune-prone strain was, in turn, compared with that in the normal strain (A/J, arbitrarily assigned a value of 1). Extracts from both lupus strains reveal a substantially reduced c-Rel to p50 ratio compared with that in A/J mice, consistent with the EMSA patterns. The levels of Rel proteins in NOD Mϕ, in contrast, do not correlate with their DNA binding activity. Normalized p65 levels are nearly identical in all strains (see *Results*).

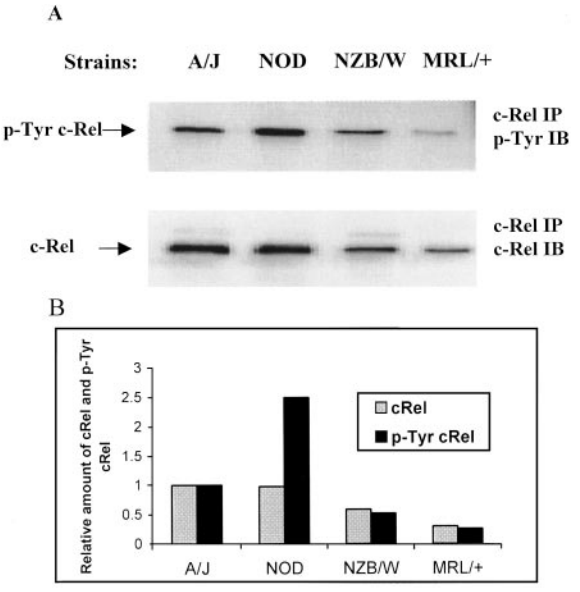

FIGURE 6. The level of c-Rel binding in NOD nuclear extracts correlates with the level of c-Rel tyrosine phosphorylation. *A*, *Upper panel*, Proteins immunoprecipitated (IP) with c-Rel Ab were separated by PAGE and then immunoblotted (IB) with anti-p-Tyr Ab. *Lower panel*, The same membrane was stripped, and immunoprecipitated with c-Rel to determine relative c-Rel levels. *B*, c-Rel and p-Tyr c-Rel were quantitated by densitometry, and normalized to the levels found in A/J (each was arbitrarily assigned a value of 1 for A/J) to determine relative expression among strains. The data show that, as shown also in Fig. 5, A/J and NOD nuclear extracts contain similar levels of c-Rel, and both are higher than c-Rel expression in lupus strains. They reveal additionally that NOD displays a unique phenotype of elevated p-Tyr c-Rel, both in absolute level and as a fraction of total c-Rel. Compared with molecular mass markers, these proteins migrated with the expected approximate molecular masses: c-Rel, 75 kDa; p50, 50 kDa; and p65, 65 kDa. The data represent one of three independent experiments that had similar results.

of the NF-κB/Rel family members. Intrinsic defects in the regulation of IL-12 may prove particularly valuable in understanding the development of autoimmunity. To our knowledge, dysregulation of IL-12 is the only protein-specific, intrinsic defect displayed in a wide range of autoimmune-prone mouse models (7–9). IL-12 plays a critical role in the selection and maintenance of the Th1 subset required for eliciting organ-specific autoimmune disease (11). For example, IL-12 blockade has been shown to inhibit the development of diabetes in the NOD mouse (19). Of note, depletion of Mϕ also blocks the development of NOD disease; diabetes is reinstated in the Mϕ-depleted NOD mouse by administering IL-12 (20), suggesting that the production of this cytokine by Mϕ is a key event in the pathogenesis of NOD disease. Thus, the specific control of IL-12 production may be important in explaining the basic dichotomy of organ-specific and system autoimmune diseases. The potential for overexpression of IL-12 may predispose diabetes-prone mice toward a Th1-mediated pathway of organ-specific autoimmunity, while reduced IL-12 in lupus-prone mice would be expected to promote B cell-mediated systemic autoimmunity. Recent reports that IL-12 inhibits B cell function in a more proximal fashion (12, 13) suggest additional mechanisms by which the deficiencies in IL-12 production in NZB/W and MRL/+ mice may contribute to B cell hyperactivity and autoantibody production. We have indeed found that coculture of B cells with Mϕ or dendritic cells from NZB/W or MRL/+ mice leads to augmented IgM production compared with coculture with APC from several normal strains (D. Alleva, J. Liu, T. Jones, and D. Beller, unpublished observations) and are currently exploring the mechanism involved.

Recently, NF-κB defects (specifically, reduced levels of p65) have been reported in T cells from lupus patients (21). Additionally, it has been shown that NOD dendritic cells overexpress κB/Rel as a consequence of enhanced function of IκB kinase (22). In distinction to our findings, the latter group reported an increase in both hetero- and homodimer binding using a consensus MHC I NF-κB sequence rather than a specific IL-12 p40 κB sequence as used here. We have found that, indeed, the hetero- and homodimers bind to a κBc site (TNF-2α) with a ratio of ~1 in A/J, NOD, and NZB/W (data not presented), suggesting that the pattern of enhanced homo- and heterodimer binding noted in the study of dendritic cells (22) is likely to reflect the κB sequence chosen, although a contribution of the cell type studied cannot be ruled out at this time. Sanjabi et al. (23) reported that while c-Rel, but not p65, was critical for regulation of Mϕ p40 expression, both Rel proteins bound the p40 κB oligo with similar affinity. This would appear to be at odds with our finding of preferential binding of c-Rel to this site. However, Sanjabi et al. (23) stimulated Mϕ with both LPS and IFN-γ, while we used LPS alone. The difference in our findings may be explained by the report by Brown et al. (24), which showed that LPS stimulation of Mϕ induced a κB-binding heterodimer comprised predominantly of c-Rel, whereas stimulation with both LPS and IFN-γ led to a heterodimer comprised of similar levels of c-Rel and p65. Taken together, these findings suggest that the precise composition of the heterodimer may be regulated by the stimulus encountered by the Mϕ. Additionally, it has been shown that 1) mutation of the 5′ region of the κB site leads to a shift in binding preference from p50/p65 to p50/c-Rel (25), and 2) mutated c-Rel proteins display unique affinities for different native κB sites (26). Thus, subtle differences in the κB

site or in flanking sequences in different promoters may also contribute to the composition of the κB/Rel heterodimer that binds this sequence.

The functional importance of our findings is supported by 1) the extraordinary correlation of the κB/Rel binding pattern with IL-12 production among a wide range of strains, 2) the documented importance of c-Rel (rather than p65) in NF-κB binding to the Mϕ p40 κB site and in the selective activation of Mϕ p40 transcription (15, 16, 23), and 3) the known inhibitory function of the p50 homodimer (27). The finding of a conserved expression of NF-κB defects in autoimmunity also may provide insight into the shared genetic loci associated with lupus, diabetes, arthritis, and experimental autoimmune encephalomyelitis (28). To our knowledge this is the first report linking selective binding of proteins from complex NF families with animal models displaying selective induction of systemic vs organ-specific autoimmunity. These findings also demonstrate the near identity in molecular regulation of Mϕ IL-12 expression in two distinct lupus models (MRL/+ and NZB/W), showing strikingly similar levels of 1) IL-12 p40 and p70 protein, 2) p40 mRNA, 3) hetero- vs homodimer binding to the p40 κB site, and 4) the ratio of nuclear c-Rel to p50. At the same time, immunoblot results provide the first evidence for unique mechanisms of regulation of the IL-12 defects among autoimmune-prone strains, revealing that, unlike the lupus strains, NOD Mϕ cannot rely solely on nuclear Rel levels to direct the hetero- and homodimer DNA binding anomaly. Enhanced phosphorylation of nuclear c-Rel may be one means by which elevated c-Rel binding occurs in NOD Mϕ. Phosphorylation regulates NF-κB activity both indirectly, via IκB degradation and release of NF-κB, and directly, through modulation of the Rel homology domain, leading to acquisition of *trans*-activating function. However, another mechanism for direct regulation of Rel family proteins and for c-Rel in particular, is phosphorylation-mediated enhancement of DNA binding function (18). c-Rel is phosphorylated after activation (29), and nonphosphorylated c-Rel does not bind to the κB site (30).

Thus, determination of the mechanisms by which 1) p50 is preferentially elevated in the cytosol or translocated to the nucleus in NZB/W and MRL Mϕ, and 2) c-Rel is more actively phosphorylated in NOD Mϕ is likely to reveal the molecular basis for inherent defects in IL-12 regulation in these strains. Taken together, these data strongly suggest that the balance of hetero- and homodimer bound to the κB site contributes to, and may determine, the level of p40 and functional IL-12 p70 production in these mouse strains. Defining the mechanisms responsible for these unique NF patterns should reveal novel molecular targets relevant to several autoimmune diseases.

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