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Rel-deficient T cells exhibit defects in production of interleukin 3 and granulocyte–macrophage colony-stimulating factor

(transcription factors)

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ABSTRACT **The c-*rel* protooncogene encodes a subunit of the NF-κB-like family of transcription factors. Mice lacking Rel are defective in mitogenic activation of B and T lymphocytes and display impaired humoral immunity. In an attempt to identify changes in gene expression that accompany the T-cell stimulation defects associated with the loss of Rel, we have examined the expression of cell surface activation markers and cytokine production in mitogen-stimulated $Rel^{-/-}$ T cells. The expression of cell surface markers including the interleukin 2 receptor α (IL-2Rα) chain (CD25), CD69 and L-selectin (CD62) is normal in mitogen-activated $Rel^{-/-}$ T cells, but cytokine production is impaired. In $Rel^{-/-}$ splenic T cell cultures stimulated with phorbol 12-myristate 13-acetate and ionomycin, the levels of IL-3, IL-5, granulocyte–macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α), and γ interferon (IFN-γ) were only 2- to 3-fold lower compared with normal T cells. In contrast, anti-CD3 and anti-CD28 stimulated $Rel^{-/-}$ T cells, which fail to proliferate, make little or no detectable cytokines. Exogenous IL-2, which restitutes the proliferative response of the anti-CD3- and anti-CD28-treated $Rel^{-/-}$ T cells, restores production of IL-5, TNF-α, and IFN-γ, but not IL-3 and GM-CSF expression to approximately normal levels. In contrast to mitogen-activated $Rel^{-/-}$ T cells, lipopolysaccharide-stimulated $Rel^{-/-}$ macrophages produce higher than normal levels of GM-CSF. These findings establish that Rel can function as an activator or repressor of gene expression and is required by T lymphocytes for production of IL-3 and GM-CSF.**

Rel/NF-κB transcription factors are homo- and heterodimeric proteins composed of subunits encoded by a small multigene family related to the c-*rel* protooncogene (1, 2). Rel/NF-κB factors regulate transcription by binding to decameric sequences (κB motifs) in the promoters and enhancers of many viral and cellular genes, in particular those that encode proteins involved in immune, acute phase and inflammatory responses (3–5). The five known mammalian Rel-related proteins, NF-κB1 (p50, p105), NF-κB2 (p52, p100), RelA (p65), RelB, and Rel share a conserved 300-amino acid N terminus [Rel homology domain (RHD)] that encompasses sequences required for DNA binding, protein dimerization, and nuclear localization (1–5). The C termini of Rel-related proteins are divergent, with those of Rel, RelA, and RelB containing transcriptional transactivation domains (1, 4). In most cells prior to stimulation, a large proportion of Rel/NF-κB is retained in the cytoplasm in an inactive form through association with regulatory IκB proteins (1–5). A wide range of stimuli promote Rel/NF-κB nuclear localization by a mechanism that involves phosphorylation and subsequent degradation of the IκB proteins (6).

Expression of the Rel subunit is largely confined to hemopoietic cells (7, 8). In the B-lymphocyte lineage, increased Rel expression results in a qualitative change in the subunit composition of Rel/NF-κB proteins during the transition from a pre-B to a B cell (9–11). A rapid increase in nuclear levels of Rel (9, 12, 13) and increased c-*rel* transcription (14) during mitogenic activation of B and T cells implicates Rel in lymphocyte proliferation. Such a function is also supported indirectly by the ability of the avian viral oncogene v-*rel* to transform both B and T cells (15). While genes regulated by Rel/NF-κB that are critical for B-cell proliferation are yet to be identified, Rel is implicated in the transcription of a number of genes that are thought to be crucial for T-cell activation (5). Rel up-regulates the transcription of human interleukin 2 receptor α (IL-2Rα) chain promoter reporters (16) and Rel/NF-κB is involved in IL-2 transcription (12, 17–19). Rel/NF-κB has also been implicated in transcriptional regulation of other cytokine genes expressed in activated T cells (5, 18). The promoters of the genes encoding IL-3, IL-5, IL-6, tumor necrosis factor α (TNF-α), granulocyte–macrophage colony-stimulating factor (GM-CSF) and γ interferon (IFN-γ) contain κB sites or the κB-related CK1/CD28 response element (CD28RE; refs. 20 and 21). The CD28RE, which is involved in IL-2 (20) and GM-CSF (22) transcription, has recently been shown to bind Rel/NF-κB complexes (21).

To better understand the physiological role of Rel, we have used gene targeting to generate mutant mice with an inactivated c-*rel* gene (19). In these mice, the development of cells from all hemopoeitic lineages appears to be normal, but humoral immunity is impaired. Mature B and T cells are largely unresponsive to a number of mitogenic stimuli and IL-2 expression is reduced in activated T cells (19). In an attempt to identify further changes in T-cell gene expression associated with the loss of Rel, we examined mitogen-activated $Rel^{-/-}$ T cells for impaired regulation of cell surface activation markers and cytokines. No differences were detected in expression of a range of cell surface markers. However, production of IL-3 and GM-CSF, but not IL-5, TNF-α, or IFN-γ, was diminished. In contrast to T cells, GM-CSF production by lipolysaccharide-stimulated $Rel^{-/-}$ resident peritoneal macrophages was higher than that of normal cells. These findings directly implicate Rel in controlling expression of a subset of cytokine genes and indicate that Rel is important for positive and negative regulation of GM-CSF expression in different cell types.

MATERIALS AND METHODS

Lymphocyte and Macrophage Activation in Tissue Culture. Splenic T cells of ≈90% purity isolated as described (19) from

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Abbreviations: IL, interleukin; TNF-α, tumor necrosis factor α; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon γ; CD28RE, CD28 response element; PMA, phorbol 12-myristate 13-acetate.

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4- to 6-week-old normal and Rel-deficient mice were stimulated for up to 72 hr in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μM 2-mercaptoethanol at a concentration of 5×10^5 cells per ml in the presence or absence of mouse recombinant IL-2 (200 units/ml; Cetus) with concanavalin A (Pharmacia) at 2 μg/ml, phorbol 12-myristate 13-acetate (PMA; Sigma) plus ionomycin (Sigma) at 2 ng/ml and 1 μg/ml, respectively, or on tissue culture plates coated with monoclonal antibodies specific for mouse CD3 (clone 145-2C11; ref. 23) and mouse CD28 (clone 37.51; ref. 24) at antibody concentrations of 10 μg/ml in the coating solution. Resident peritoneal macrophages isolated from 6- to 8-week-old mice were first purified by adherence to plastic dishes for 3 hr and then cultured at 5×10^5 cells per ml in Dulbecco's Modified Eagles medium containing 10% fetal calf serum and lipopolysaccharide at 0.1 or 1 μg/ml for 24–48 hr.

Immunofluorescence Staining and Flow Cytometry. The expression of the IL-2Rα chain (CD25), CD69, and L-selectin (CD62) were analyzed on T cells before and after 24, 48, and 72 hr of stimulation. Cells were stained as described (25) with anti-Thy1.2-FITC (clone 30H12; Becton Dickinson) and with biotinylated monoclonal antibodies specific for IL-2Rα chain (clone PC61), CD69 (clone H1.2F3), or CD62/L-selectin (clone Mel-14) or with a biotinylated isotype-matched control monoclonal antibody. Binding of biotinylated antibodies was revealed by staining with phycoerythrin/streptavidin (Caltag) as the secondary reagent.

Cytokine Assays. The levels of IL-3, IL-5, TNF-α, and GM-CSF in T-cell culture supernatants were determined by ELISA (Pharmingen, San Diego, CA) according to the manufacturer's instructions. IL-3 levels were also determined by bioassay using IL-3-responsive 32D cells (26), and the level of IFN-γ was detected by using the IFN-γ sensitive cell line WEHI279 as described (27). GM-CSF levels produced by stimulated peritoneal macrophages were determined by bioassay as described (28). All cytokine assays were standardized by including a titration of the appropriate purified recombinant cytokine of known activity.

RESULTS

The Expression Pattern of T Cell Surface Markers Is Normal in Mitogen-Activated Rel-Deficient Cells. Although expression of a wide range of cell surface markers was shown to be normal in naive Rel-deficient T cells (19), a lack of response to certain modes of activation prompted a study of surface marker expression during mitogenic stimulation. Changes in expression of a number of surface markers normally occurs with the onset of T-cell activation. CD69 (29), CD25/IL-2Rα chain (30), CD44/Pgp1 (30), and the Fas/Apo-1 (CD95) receptor (31, 32) are all up-regulated during T-cell stimulation, while L-selectin/CD62 (33) and the CD45 isoform CD45RB (33) are down-regulated. Flow cytometry was used to monitor expression of these cell surface markers in normal and in $Rel^{-/-}$ T cells stimulated over a 72-hr time course with PMA and ionomycin or with anti-CD3 and anti-CD28. A representative sample of results from these experiments (Fig. 1) clearly establishes that 72 hr poststimulation the expression of CD25, CD69, and MEL14 in response to both sets of stimuli is normal. An examination of these and other cell surface markers after 24 and 48 hr of stimulation confirmed that the kinetics of expression on $Rel^{-/-}$ T cells was also normal (results not shown). These results therefore document that Rel is not essential for expression of these proteins.

The Expression of IL-3 and GM-CSF Is Reduced in Mitogen-Stimulated Rel-Deficient T Cells. Activated T cells secrete a number of cytokines that are critical in modulation of immune responses (34). The finding that $Rel^{-/-}$ T cells make reduced levels of IL-2 (19) prompted us to compare the cytokine secretion profile of normal and $Rel^{-/-}$ T cells stimulated with different mitogenic agents for 72 hr (Table 1). Cytokine levels were consistently lower in stimulated $Rel^{-/-}$ T cell cultures, with the relative difference in expression of each cytokine dependent on the stimulus. For example, while cytokine levels in PMA plus ionomycin-stimulated $Rel^{-/-}$ T cells were equivalent to or only severalfold lower than in normal cultures, cytokine levels in anti-CD3 plus anti-CD28-stimulated $Rel^{-/-}$ T cells were reduced 12- and ≈80-fold for GM-CSF and IL-3, respectively.

The poor proliferative response of anti-CD3 plus anti-CD28-stimulated $Rel^{-/-}$ T cells compared with that elicited by PMA and ionomycin (19) prompted an examination of cytokine production by mitogen-stimulated cultures supplemented with IL-2. Addition of IL-2, which restores $Rel^{-/-}$ T cell proliferative responses to that of normal cells (19), should distinguish between reduced cytokine production due directly to the loss of Rel or as a secondary effect resulting from the lack of proliferation. These experiments (Table 1) establish that defects in cytokine production by $Rel^{-/-}$ T cells fall into two groups. The amount of IFN-γ, TNF-α, and IL-5 now produced by stimulated $Rel^{-/-}$ T cells was similar to that of normal cells, indicating that reduced levels of these cytokines appear to be due to the impaired proliferative response. In contrast, the amount of IL-3 and GM-CSF produced by anti-CD3 plus anti-CD28-stimulated $Rel^{-/-}$ cells supplemented with IL-2 was still 16- and 11-fold lower, respectively, than that made by normal T cells, indicating that Rel was essential for normal expression of these cytokines.

Rel Has Opposite Effects on Regulation of GM-CSF Expression in T Cells and Macrophages. GM-CSF can be produced not only by activated T cells but also by a number of cell types including fibroblasts, macrophages, and endothelial cells in response to stimulation by cytokines, antigens, or inflammatory agents (35). Since Rel expression is largely confined to hemopoietic cells (7, 8), GM-CSF production was also examined in macrophages from $Rel^{-/-}$ mice. In contrast to the reduced GM-CSF levels secreted by mitogen-stimulated $Rel^{-/-}$ T cells, resident $Rel^{-/-}$ peritoneal macrophages treated with lipopolysaccharide for 24 or 48 hr produce 10- to 50-fold more GM-CSF than normal macrophages (Fig. 2). This did not appear to be a lipopolysaccharide concentration-dependent phenomenon, since 0.1 or 1 μg/ml of lipopolysaccharide per ml both resulted in elevated production of GM-CSF from $Rel^{-/-}$ macrophages. The basis for the slight reduction in GM-CSF levels produced by both the normal and $Rel^{-/-}$ cultures after 48 hr stimulation compared with that seen after 24 hr most likely reflects GM-CSF consumption by the macrophages. Consistent with the levels of GM-CSF determined by bioassay and ELISA, Northern blot analysis confirmed that GM-CSF mRNA levels were reduced in $Rel^{-/-}$ T cells and elevated in $Rel^{-/-}$ macrophages compared with their normal counterparts (S.G. and G.G., unpublished results). Collectively, these results establish that Rel can function as both an activator and repressor of induced GM-CSF expression in different activated cell types.

DISCUSSION

Changes in expression of many genes accompany T-cell activation (36). The finding that T cells from $Rel^{-/-}$ mice exhibit defects in proliferation and reduced IL-2 expression (19) prompted a detailed study of gene expression during the mitogenic activation of $Rel^{-/-}$ T cells. The expression pattern of cell surface markers including CD25, CD44, CD45RB, CD69, and CD95, all of which normally change during T-cell activation (29–33), were unaffected in $Rel^{-/-}$ T cells. The observation that Rel is not essential for basal or induced IL-2Rα chain gene expression in murine T cells was surprising. Although Rel binds to a κB site in the promoter of the human IL-2Rα chain gene required for Rel-dependent transactivation

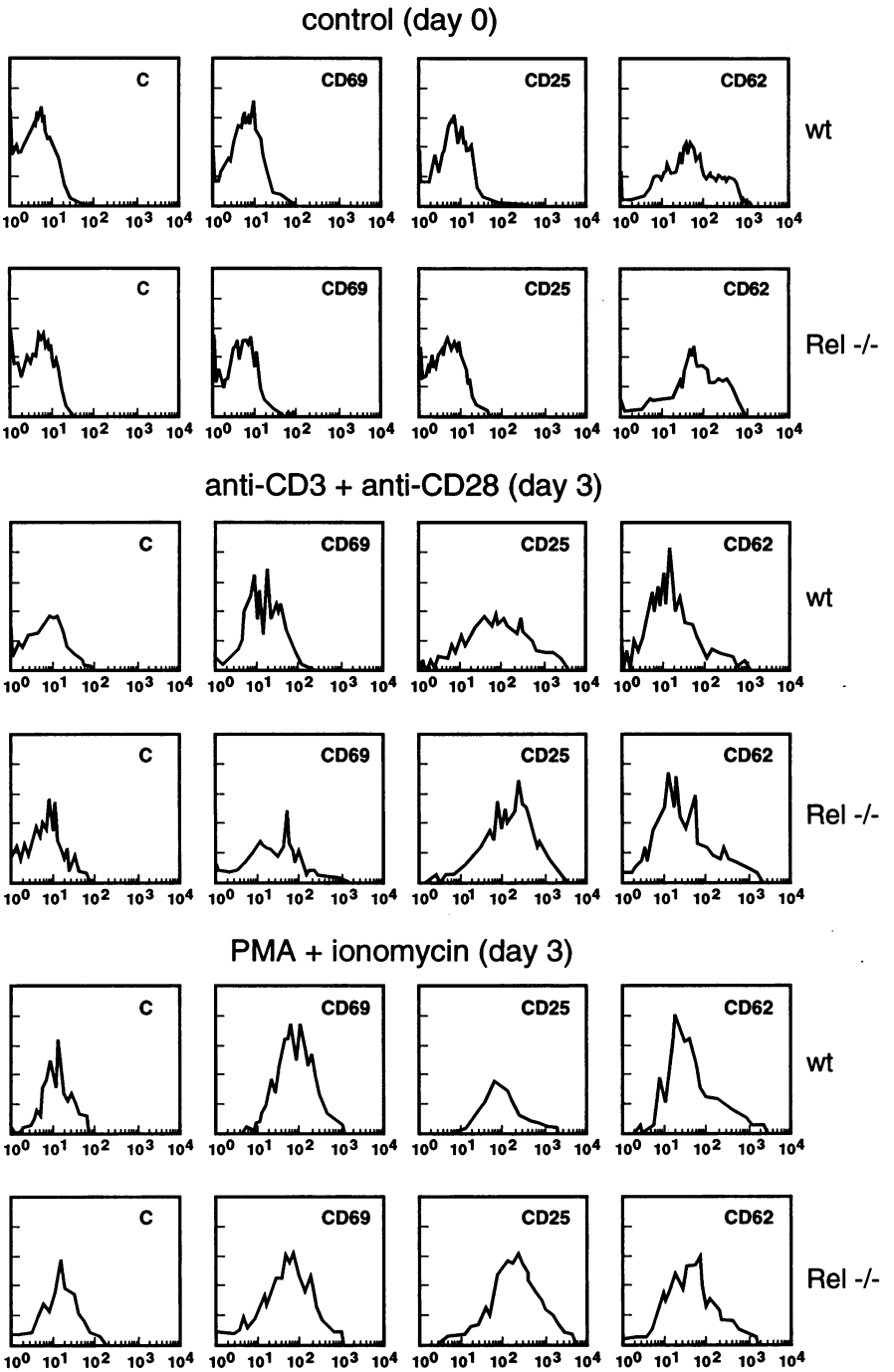

FIG. 1. Expression of cell surface activation markers on T cells from Rel$^{-/-}$ mice. Spleen cells from normal and Rel$^{-/-}$ mice were stimulated in tissue culture either with immobilized anti-CD3 plus anti-CD28 or with PMA plus ionomycin. Expression of IL-2Rα chain (CD25), CD69, and L-selectin (CD62) was analyzed on T cells (identified as being Th1.2 positive) before and after 72 hr of stimulation. Data are representative of three independent experiments and are presented as histograms of fluorescence intensity of Thy1.2-positive gated T cells.

of reporter constructs (16) and v-*rel* upregulates avian IL-2Rα chain expression in Rev-T-transformed lymphoid cells (37), a comparison of mouse and human IL-2Rα chain gene promoter sequences (38) may explain the basis of this unexpected species-specific difference. The κB site in the human IL-2Rα chain promoter, 5′-GGGAATCTCC-3′, differs from the sequence in the mouse gene, 5′-GGAATCCCCC-3′, the latter of which is a poor consensus binding site for Rel/NF-κB proteins (1, 3, 39). Collectively, these findings suggest that Rel is not important in murine IL-2Rα chain gene expression.

In contrast to the regulation of cell surface markers, cytokine gene expression is perturbed in Rel$^{-/-}$ T cells. The levels of IL-2 (19), IL-3, IL-5, GM-CSF, TNF-α, and IFN-γ were low or undetectable in concanavalin A or anti-CD3 plus anti-CD28-stimulated Rel$^{-/-}$ T-cell cultures. Rescuing the proliferation of Rel$^{-/-}$ T cells by adding IL-2 revealed that IL-5, TNF-α, and IFN-γ production was restored to normal levels, but expression of IL-3 and GM-CSF remained much lower than in the normal T-cell cultures. These findings indicate that in this system expression of some but not all cytokine genes appear to be directly controlled in part by Rel and that cytokine production rather than cell surface marker expression may be a more accurate gauge of T-cell activation. While the ability of exogenous IL-2 to restitute normal expression of

Table 1. Cytokine production by Rel$^{-/-}$ T cells in response to different mitogenic stimuli

Stimulus	IL-3, pg/ml		IL-5, pg/ml		GM-CSF, pg/ml		TNF-α, pg/ml		IFN-γ, ng/ml	
	+/+	−/−	+/+	−/−	+/+	−/−	+/+	−/−	+/+	−/−
None	<30	<30	<30	<30	<30	<30	<30	<30	<1	<1
IL-2	<30	<30	<30	<30	<30	<30	<30	<30	<1	<1
Concanavalin A	600 ± 70	<30	110 ± 30	<30	625 ± 80	120 ± 30	315 ± 50	175 ± 40	20	ND
Concanavalin A + IL-2	1,350 ± 110	65 ± 20	190 ± 40	160 ± 40	1025 ± 110	170 ± 20	480 ± 70	350 ± 50	20	15
PMA + ionomycin	75,000 ± 5500	23,500 ± 2100	410 ± 60	250 ± 30	5600 ± 240	2350 ± 180	1670 ± 180	910 ± 110	80	80
PMA + ionomycin + IL-2	68,000 ± 4000	29,000 ± 1500	520 ± 40	470 ± 50	7150 ± 570	2650 ± 190	2240 ± 270	1820 ± 210	80	80
Anti-CD3 + anti-CD28	34,500 ± 2500	450 ± 70	340 ± 30	<30	4800 ± 230	350 ± 60	2750 ± 230	130 ± 40	160	20
Anti-CD3 + anti-CD28 + IL-2	42,000 ± 3100	2,600 ± 160	480 ± 40	540 ± 60	7100 ± 480	650 ± 80	3060 ± 240	3160 ± 190	160	>300

Specific cytokine levels produced by T cells stimulated for 72 hr were determined by comparing supernatant activities with those of purified recombinant cytokine standards and were converted to pg/ml or ng/ml. Means ± SD for IL-3, IL-5, GM-CSF, and TNF-α were determined from four to six separate experiments. IFN-γ levels are means of two experiments. <, Cytokine activities lower than the limit of sensitivity for detection by either cytokine ELISA or bioassay; ND, not determined.

certain T-cell cytokines is consistent with cytokine production being intimately linked to proliferation, it remains a formal possibility that the IL-2-mediated rescue of IL-5, IFN-γ, and TNF-α expression in anti-CD3 plus anti-CD28-treated Rel-deficient T cells is associated with an aspect of T-cell activation that is independent of the IL-2-dependent proliferative response.

The transcriptional control regions of the IL-2, IL-3, and GM-CSF genes share a number of regulatory sequences including the CK1 or CD28RE, which is distinct from but related to the κB motif and has been shown to bind complexes containing NF-κB1, RelA, and Rel (21). A compilation of CD28REs within cytokine gene promoters is shown in Fig. 3. The GM-CSF motif is a perfect match with the Rel homodimer consensus binding site 5′-NGGNNA/TTTCC-3′ (39), while the sequences from the IL-2, IL-3, and G-CSF genes differ by only a single nucleotide from the consensus motif. Although the IFN-γ CD28RE differs by only 2 nucleotides from the consensus sequence, these changes reside in the 3′ half-site, which is the most conserved region of the Rel binding element (39). Therefore, the ability of Rel to preferentially bind to particular CD28REs may account for the relative defects in expression of the different cytokine genes in the Rel$^{-/-}$ T cells.

Despite evidence that phorbol ester and calcium ionophore treatment mimics activation mediated through cross-linking of the T-cell receptor and CD28 costimulatory receptor (18), the relative levels of IL-3 and GM-CSF produced by normal and Rel$^{-/-}$ T cells in response to these two stimuli differ. The ability of PMA and ionomycin to induce IL-2 (19), IL-3, and GM-CSF, albeit several fold less than in control cells in the absence of Rel, may indicate that in T cells, Rel is either upstream of the point at which these agents engage a common intracellular pathway required for the transcription of these genes or that these agents operate through a distinct pathway. Alternatively, PMA and ionomycin, but not anti-CD3 plus anti-CD28 costimulation may activate other Rel family proteins or different transcription factors that substitute in part for Rel.

A comparative analysis of GM-CSF production by mitogen-activated Rel$^{-/-}$ T cells and macrophages indicates that Rel can function both as an activator or repressor of gene expression. While this dual activity is well established for the *Drosophila* Rel homologue Dorsal (40, 41), the results presented here are the first clear demonstration that a mammalian

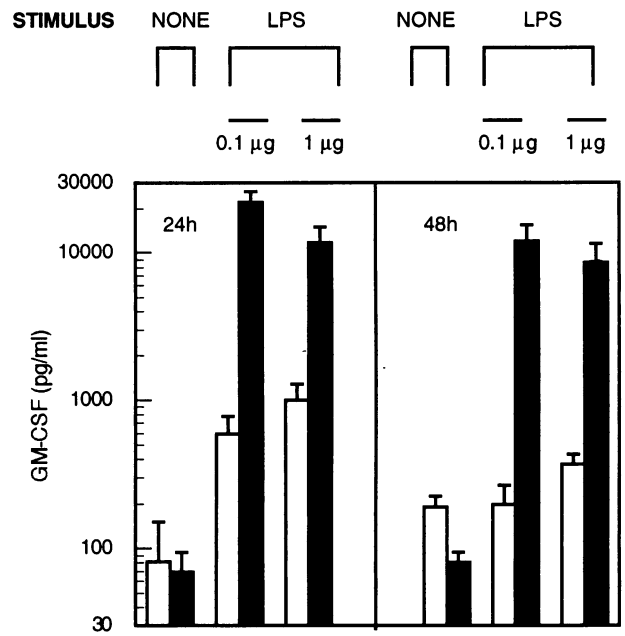

FIG. 2. Level of GM-CSF produced by lipopolysaccharide (LPS)-activated Rel$^{-/-}$ peritoneal macrophages is elevated compared with normal cells. Resident peritoneal macrophages from normal (open bars) or Rel$^{-/-}$ (solid bars) mice were stimulated with lipopolysaccharide (0.1 or 1 μg/ml) for 24 and 48 hr. Each histogram represents the mean ± SE from four or six experiments.

GENE	CD28RE SEQUENCE	LOCATION
IL-2	A a G A A A T T C C	-163 to -154
GM-CSF	A G G A G A T T C C	-109 to -98
IL-3	T G G A G g T T C C	-116 to -105
G-CSF	C a G A G A T T C C	-193 to -182
INF-γ	A G G A A A c T C t	-171 to -160
Consensus Rel binding motif	5′-N G G N N A/T T T C C-3′	

FIG. 3. Compilation of CD28 response elements in the promoters of murine cytokine genes. These sequences are compared with the consensus Rel binding site (39), and nonconserved bases are shown in lowercase. Nucleotide numbering assigned to these elements in the 5′ flanking sequence of the different mouse cytokine genes is according to the start sites of transcription.

Rel protein possesses these properties. One plausible model that could explain how Rel is an activator of GM-CSF expression in T cells but a repressor of the same gene in macrophages is the modulation of Rel activity through interaction with different transcription factors in the two cell types. This model is consistent with the ability of Rel/NF-κB proteins to associate with a number of transcription factors including ATF-2 (42), SP1 (43), and the C/EBP proteins (44). The finding described here could result from Rel associating with different Rel-like subunits or with unrelated proteins. For example, different high mobility group (HMG) proteins can determine whether Rel/NF-κB factors function as activators or repressors of transcription. Binding of HMG I (Y) at sites adjacent to the κB element in the IFN-β gene augments both NF-κB binding and transcriptional activity (45), while the DSP1 HMG protein binds to a negative regulatory element flanking the IFN-β κB site and converts NF-κB from a transcriptional activator to a repressor (46). The identification of genes whose expression is induced or repressed by Rel should provide a basis for elucidating the mechanism(s) governing the transcription regulatory properties of Rel/NF-κB factors.

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