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Differential Effects of Interleukin-1β, Interleukin-2, and Interferon-γ on the Inducible Expression of CYP 1A1 and CYP 1A2 in Cultured Rabbit Hepatocytes

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The effects of interleukin-1β, interleukin-2 and interferon-γ and their combinations were investigated on induced cytochrome P 4501A of cultured rabbit hepatocytes considered 72 h after plating. Without apparent cellular toxicity, these cytokines provoke a significant decrease in TBZ- and BNF-induced P4501A1/2 expression. However specific patterns of action are revealed : IL-1β is the most potent cytokine in regard to CYP1A1/2 mRNA suppression whereas IL-2 exerts repressive effects only on P4501A1 induced expression. Although being a strong inhibitor of induced enzymatic activities and protein contents, IFN-γ exhibits only a weak influence on CYP1A1/2 mRNAs with the exception of its association with interleukins. All these results suggest that IL-1β and IL-2 promote mainly a transcriptional repression mechanism whereas IFN-γ would stimulate a post-transcriptional suppressive pathway.

Inhibition of xenobiotic biotransformations is a well-known detrimental consequence of infection with bacteria, viruses or parasites in humans and animals (1, 2, 3). More specifically, immunological and inflammatory stimuli have been shown to induce a deep depression of cytochrome P450-mediated hepatic metabolism in numerous *in vivo* and *in vitro* experiments (2). Previous data demonstrate that several inflammatory cytokines, notably IL-1β and IFN-γ, the principal mediators of the liver acute-phase response, are major contributors to the decline of hepatic P450 activity and content (for a review, see Ref. 4). By contrast, the few results concerning the effects of IL-2, the main lymphocyte activator, remain controversial. Recently, *in vivo* administration of IL-2 was reported to decrease several monooxygenases activities in mouse (5). On the contrary, a low dose of rat IL-2 was shown to increase slightly P450-related activities after *in vivo* administration to rats (6). Finally, *in vitro* study demonstrated the presence of the IL-2 receptor in hepatocytes, as already described for IL-1β and IFN-γ (7, 8), and its potential down-regulating capability on the constitutive expression of P450 genes in cultured rat hepatocytes (9). However, no comprehensive analysis of *in vitro* effects of IL-2 on the inducible expression of P450 has never been reported. So, using primary cultures of rabbit hepatocytes, our approach focused on the influence of IL-2 alone or in combination with IFN-γ on induced P4501A expression and activities, one of the major inducible P450 family in rabbit (10). The effects of IL-1β, IFN-γ and their association were also investigated, with the intention of comparing the repression pattern of the various cytokines treatments. Moreover, considering that CYP1A induction relies on two distinct mechanisms (ligand-dependent or -independent activation of Ah receptor), the influence of both inducing pathways on the subsequent cytokine effects was determined by using two different CYP1A inducers : a polycyclic aromatic hydrocarbon, BNF and a benzimidazole derivative TBZ, two chemicals well known to increase transcription of P4501A subfamily through a ligand-dependent (BNF) or -independent (TBZ) activation of Ah receptor (11, 12).

MATERIALS AND METHODS

Materials. TBZ, BNF, ethoxyresorufin, methoxyresorufin, DMSO, Waymouth, Ham F12 and William's E culture media, horseradish

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Abbreviations: IL-1β, interleukin-1β; IL-2, interleukin-2; IFN-γ, interferon-γ; P450, cytochrome P450; CYP1A1, gene encoding for P4501A1 isoenzyme; TBZ, thiabendazole; BNF, β-naphtoflavone; EROD, ethoxyresorufin O-deethylase; MROD, methoxyresorufin O-demethylase; LDH, lactate dehydrogenase; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde phosphate dehydrogenase; FCS, fetal calf serum; SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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peroxidase antibodies, collagen, collagenase type IV and BSA were obtained from Sigma (Saint Quentin, France). Human recombinant IL-1β and IFN-γ were purchased respectively from Boehringer Mannheim and PeproTech EC Ltd. (London, England). Human recombinant IL-2 was a generous gift from Dr. Martini (Roussel-Uclaf, Romainville, France). Antirabbit P4501A1/2 IgG and cDNA probes were generous gift from Dr. P. Maurel (INSERM, Montpellier, France) and F.J. Gonzalez (NIH, Bethesda, USA). FCS was from Biochrom KG (Berlin, Germany). All other chemicals used were of the highest purity commercially available and distilled deionized water was used in all studies.

Hepatocyte culture and drug treatment. Male New-Zealand rabbits were obtained from INRA Elevage Lapin (Toulouse, France). Hepatocytes were isolated and cultured as already described (13). For cytokine treatments, the lowest concentrations previously found to regulate *in vitro* CYP expression (9, 14, 15) were used. So IL-1β, IL-2 and IFN-γ were dissolved in pure water and distributed in the culture medium without FCS at final concentrations of respectively 2 U /mL, 5000 U /mL and 50 U /mL, 72 h after plating in order to avoid post-plating perturbations. Four hours after cytokine input, BNF or TBZ dissolved in DMSO, were added at a final concentration of respectively 30 μM and 200 μM. Treatments lasted for 20 h, control plates receiving only DMSO (1% final volume). Microsomal fractions were classically prepared by differential centrifugation of the sonicate of three pooled cultures (16). Proteins were determined according to Lowry (17) using BSA as standard.

Biochemicals assays. Cytotoxicity resulting from treatment of hepatocytes was assessed by the titration of LDH in both cytosolic fraction and culture medium (18). Total microsomal cytochrome P450 content and the *O*-dealkylations of ethoxyresorufin and methoxyresorufin were classically determined (19, 20). As an oxidation product of NO biosynthesis, nitrite was determined spectrophotometrically at 540 nm, based on the diazotization assay, generally referred to as a Griess reaction (21).

Western blot and Northern blot analysis. Microsomal proteins (5 μg) were resolved on a 10% SDS-PAGE (22), electrophoretically transferred to nitrocellulose sheet and developed by specific IgG as previously described (23). Total RNA was isolated (24) and 10 μg were size-fractionated on a 1,2 % agarose gel and blotted onto Gene Screen Plus hybridation transfert membrane (Dupont, Albany, USA). Hybridization (25) was performed in the presence of appropriate probes (CYP1A1, CYP1A2 and GAPDH as control) radiolabeled with the Ready-to-go kit (Pharmacia Biotech, Saclay, France).

Analysis of data. Relative content of each isoenzyme and mRNA were estimated by densitometry of the western blots and northern blots by using an omnimedia XRS 12CX Bioimage scanner (Roissy, France). Statistical significance was evaluated using analysis of variance including a complementary range test in order to compare means. In all the cases, a probability of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

As demonstrated by the unchanged values of LDH leakages (4-5 %) and of microsomal proteins (2.2-2.5 mg /ml), all cell treatments including TBZ or BNF induction, appeared to be devoid of any cytotoxic effect. In the meantime, the significant equal increases in NO release indicated clearly hepatocyte responsiveness to all investigated cytokines (26) in our culture conditions, containing less than 5 % of non-parenchymal cell contamination (Figure 1). Significant inductions (1.6-1.7-fold / control) of total P450 were obtained in presence of TBZ and BNF but cytokines did not affect this parameter (Table 1), suggesting a selective cytokine action, if any, on certain P450 isoenzymes. Incubation with TBZ or BNF led to a 4-fold increase of the dependent P4501A1 EROD activity (27). In cytokine-treated cells, BNF induction was strongly reduced (Table 1) but IL-2 remained significantly less active in inhibiting BNF-induced EROD (25 % decrease) than IL-1β (30 %) and IFN-γ (42 %). By contrast, in TBZ-induced samples, significant EROD decrease appeared only in presence of IL-1β (about 45 % decrease). Cytokine associations did not provoke any significant additional decrease in EROD induced activity. In parallel, MROD, often described to be more representative of P4501A2 isoenzyme activity (27), was significantly induced by TBZ and BNF (about 2-fold /control). Its resistance to cytokine treatment was also observed in case of TBZ induction. But even in the presence of BNF, IL-2 did not impair this activity, whereas IL-1β, IFN-γ and the two combinations significantly reduced MROD, with almost equivalent efficiencies (about 40 % decrease) (Table 1).

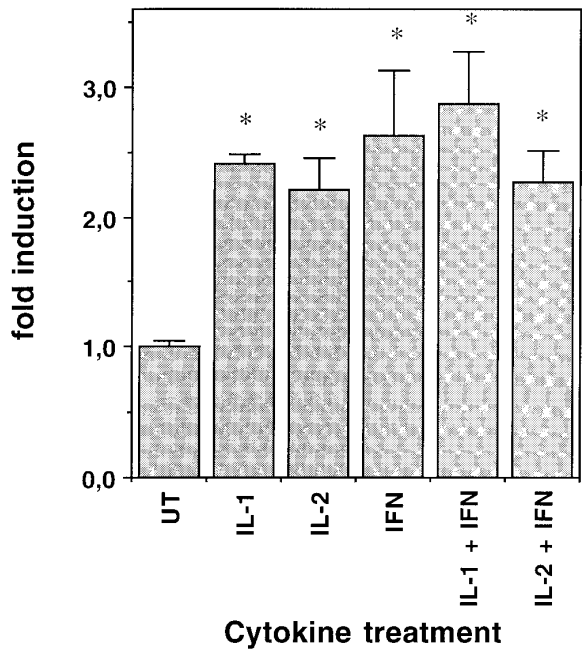

FIG. 1. Induction of NO biosynthesis in rabbit hepatocytes by human recombinant cytokines. Hepatocytes were exposed to the indicated treatments for 24 h. The final cytokine concentrations were respectively 2 U /mL for IL-1β, 5000 U /mL for IL-2 and 50 U /mL for IFN-γ. Nitrite was measured in the culture supernatants as a stable endproduct of NO generation. Results are expressed as induction factors of cytokine-treated samples vs DMSO-treated control (UT). The values represent the means $\pm$ SD of two distinct experiments measured in duplicates. * Significant increase due to cytokine, relative to untreated culture ($P < 0.05$).

The relative amounts of specific CYP1A proteins were determined by immunoblot analysis. In agreement with previous reports (10, 12), BNF or TBZ treat-

TABLE 1

Effect of Cytokines on Induced Total P450 and P4501A-Related Monooxygenases in Rabbit Hepatocyte Cultures

Condition	Induction	Total P450 (nmol/mg)	EROD (pmol/min/mg)	MROD (pmol/min/mg)
Control	—	0,35 ± 0,02	13,1 ± 0,2	19,8 ± 1,9
	TBZ	0,55 ± 0,03[a]	50,7 ± 2,3[a]	39,2 ± 4,8[a]
	BNF	0,59 ± 0,01[a]	55,0 ± 2,9[a]	39,8 ± 3,0[a]
IL-1β	TBZ	0,58 ± 0,06	33,1 ± 0,9*	31,3 ± 0,2*
	BNF	0,54 ± 0,06	38,9 ± 0,1*	30,0 ± 0,3*
IL-2	TBZ	0,58 ± 0,05	46,9 ± 0,9	36,0 ± 0,5
	BNF	0,56 ± 0,10	47,1 ± 0,3*	34,8 ± 1,4
IFN-γ	TBZ	0,49 ± 0,15	47,1 ± 5,5	40,7 ± 4,5
	BNF	0,57 ± 0,04	30,7 ± 8,1*	22,8 ± 5,6*
IL-1β+IFN-γ	TBZ	0,46 ± 0,13	25,4 ± 1,1*	25,5 ± 0,1*
	BNF	0,52 ± 0,03	27,5 ± 1,2*	21,0 ± 0,9*
IL-2+IFN-γ	TBZ	0,54 ± 0,04	48,1 ± 0,6	37,5 ± 0,2
	BNF	0,51 ± 0,06	26,3 ± 3,9*	18,4 ± 2,8*

All values are means ± SD from three different cultures measured in duplicates.
[a] Significant differences ($p < 0.05$) from control cultures receiving only DMSO.
* Significant differences ($p < 0.05$) from TBZ- or BNF-induced control cultures.

ment resulted in a significant increase of CYP1A1 protein level (about 7-8-fold /control) (Figure 2A). All cytokine treatments decreased the level of induction of CYP1A1 whatever the inducer was. However IL-2 and IFN-γ were significantly more potent (30 to 61 % decrease) than IL-1β). Moreover, BNF-induced CYP1A1 protein content correlated ($r = 0{,}988$) with EROD activity for IL-2, IFN-γ and IL-2+IFN-γ treatments. By contrast, addition of IL-1β provided a more marked decrease of EROD activity than that of CYP1A1 protein level. Again, no additive effect was revealed in the case of cytokine combinations. BNF exposure led to a moderate but significant increase (about 1,3-fold /control) of CYP1A2 protein level (Figure 2B), whereas TBZ remained ineffective. This weak inducibility was associated with a relatively strong resistance to cytokine treatments, since the only IFN-γ was able to decrease P4501A2 apoprotein expression by 30 %. But unexpectedly, IL-2 significantly potentiated IFN-γ action so that this combination depressed CYP1A2 induced protein levels by 42 % and 57 % respectively, with TBZ or BNF (Figure 2B). Consequently, in the presence of BNF, P4501A2 protein content correlated ($r = 0{,}980$) with MROD activity in case of IL-2, IFN-γ and IL-2+IFN-γ treatments. In addition, IL-1β produced a significant alteration of MROD activity without any effect on corresponding protein level. This phenomenon was already noticed, although less pronounced, for CYP1A1.

Finally, the reduction of P4501A1/2 expression by cytokines was investigated via northern blotting analysis. As previously reported (10), basal CYP1A1 and 1A2 mRNAs remained undetectable whereas TBZ and BNF induced both messagers with a slightly better efficiency regarding BNF. Densitometric analysis including normalization to GAPDH mRNAs demonstrated that IL-1β, IL-2, IFN-γ, IL-1β+IFN-γ and IL-2+IFN-γ decreased CYP1A1 mRNA by respectively 45 %, 49 %, 38 %, 52 % and 59 % in case of TBZ induction. The corresponding values, relative to BNF induction, were respectively 22 %, 25 %, 16 %, 25 % and 14 % (Figure 3A). Obviously, the degree of mRNA reduction was strikingly more important in presence of TBZ than of BNF whereas IFN-γ was significantly less efficient than both interleukins. Moreover, P4501A1 protein level was less affected than CYP1A1 mRNA for IL-1β and IL-2 treatments in case of TBZ induction. An opposite situation appeared with IFN-γ, due to its limited influence on CYP1A1 induced mRNAs. But IL-2 + IFN-γ association resulted in a significant additional effect in presence of TBZ, in comparison with IL-2 and IFN-γ alone. In parallel, CYP1A2 mRNAs were also significantly reduced by 40 % and 55 % in the presence of respectively IL-1β and IL-1β+IFN-γ whatever the inducer was. And IL-1β produced a more important reduction of P4501A2 mRNA than of corresponding protein level. But all other cytokine treatments remained ineffective (Figure 3B) with the exception of a significant potentiating action of IFN-γ on IL-1β. No regulation of GAPDH mRNA by the different treatments was observed (Figure 3C), showing that all these changes were not based on unspecific effects of cytokines, like a decrease of cell viability.

The results of the present study, using rabbit hepatocytes, indicate a specific role for each cytokine in regulating induction of P4501A1/2, although differing in their activity. Besides the fact that IL-2 remains weakly efficient, in regard to EROD activity, IL-1β and IL-2 are almost equipotent in inhibiting CYP1A1 induced expression. By contrast, IL-2 is ineffective in reducing P4501A2 induced expression whereas Il-1β

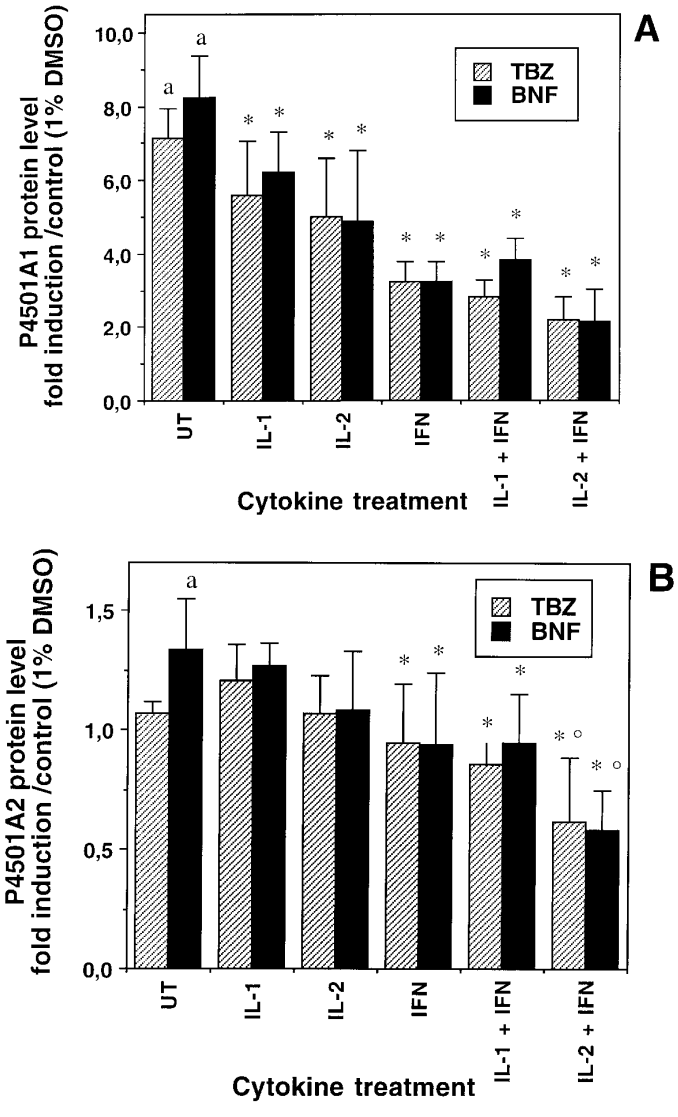

FIG. 2. Effect of cytokines on induced P4501A1 and 1A2 protein levels. Treatments and immunoblotting analysis were performed as described in Materials and Methods. Relative P450 protein amounts were determined by densitometry. Data are expressed as induction ratios between samples treated with inducers alone (UT) or together with cytokines and untreated control (1% DMSO). Incubations with TBZ are given in the *hatched columns* whereas *black columns* represent incubations with BNF. The values correspond to the means ± SD of four distinct experiments measured in duplicates. **A.** P4501A1 protein level, **B.** P4501A2 protein level. [a] significantly different from untreated control (1% DMSO) ($P < 0.05$). * significantly different from corresponding induced control (UT) ($P < 0.05$). ° significantly different from single cytokine treatments ($P < 0.05$).

stongly suppress CYP1A2 mRNA without any change in corresponding protein level and activity. The degree of interleukin-mediated reduction of CYP1A1/2 mRNA is generally more pronounced than the diminution of the respective protein levels. This phenomenon may probably reflect the short duration of experiments (24 h), which does not allow to reach maximal protein decrease, considering P4501A protein turn-over (about 20 h) (10). IFN-γ provides the most potent decrease of both P450 1A1/2 activities and their corresponding protein levels but exerts little or no influence on CYP1A1/2 mRNAs. In the same way, the association of IL-2 and IFN-γ exhibits significant additional repression on induced 1A2 protein content but not on P450 1A2 messagers. By contrast, the same combination provides an additive effect on CYP1A1 mRNA which does not appear for the corresponding protein level. IL-1β+IFN-γ association results also in a significant synergistic decrease only on CYP1A2 mRNA. CYP1A2 induced expression is known to be mainly under post-transcriptional control, whereas CYP1A1 induction has been shown to be transcriptionally regulated (10). So, IL-2 would act probably at a transcriptional level, either by decreasing transcription rate or by increasing mRNA degradation. Decrease of transcription rate is also probably involved in repressive action of IL-1β, as it has already been described for this cytokine (14). However the higher reduction of enzyme activities by comparison to corresponding protein contents would suggest a possible functional inhibition of P450 catalysis. In fact, IL-1β could provoke increased heme loss, since it has been shown to induce heme oxygenase which is responsible for P450 heme degradation (28). In parallel, the major post-transcriptional action of IFN-γ would be mediated either by enzyme degradation via its well-known induction of xanthine oxidase, which subsequently generates the free radicals that destroy P450 apoprotein, or by reduction of P450 mRNA translation (29, 30). Then, cytokine associations lead to cumulative effects, via the combination of various potential pathways, including likely mechanisms that remain ineffective when cytokines are used separately. On the other hand, the common capability of cytokines to induce NO synthesis do not account for the differential effects observed, suggesting that in our experimental conditions, NO release does not play an important role, if any, in the repression of P4501A expression. This results is unconsistant with previous reports describing this small reactive molecule as a potent mediator of the decrease in P450-dependent metabolism caused by immunostimulants (31). This difference could be related to the low cytokine concentrations used in our study as compared to those classically used in other *in vitro* investigations (32). Interestingly, the induction protocol seems also to influence the observed responses. In absence of cytokine, considering the used concentrations, BNF seems to be a more potent inducer of CYP1A1/2 than TBZ. Then a higher level of transcriptional activation due to its presence could explain the partial reduction of cytokine inhibitory effects that we noticed on CYP1A1 mRNAs. These two remarks indicate that there is probably a relationship between the ligand-dependent or -independent activation of Ah receptor and the cytokine effects, through the intensity

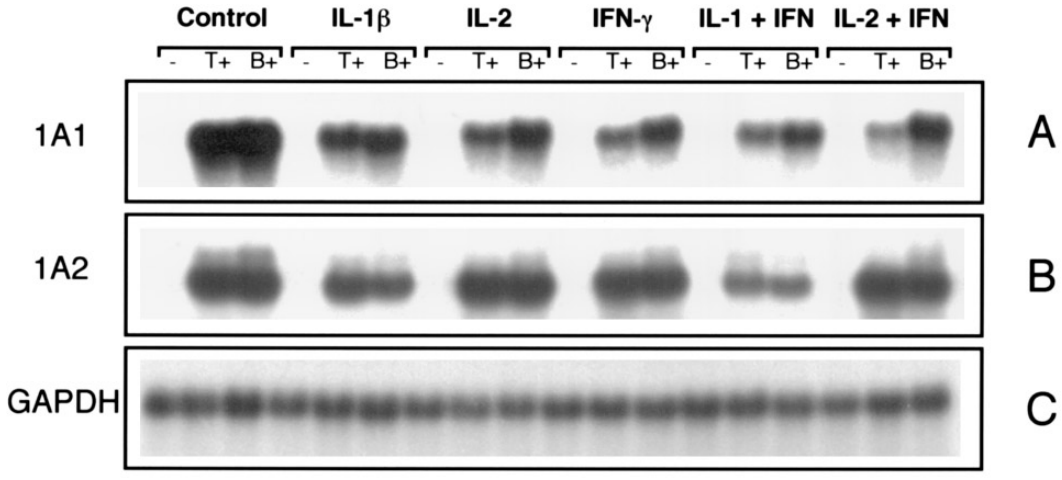

FIG. 3. Effect of cytokines on induced CYP1A1 and CYP1A2 mRNAs. Rabbit hepatocytes were incubated with indicated treatments during 24 h, as described in Materials and Methods. Then, total RNAs (10 μg) were subjected to Northern blot analysis. Membranes were hybridized with ^{32}P-labeled cDNA probes, respectively CYP1A1 (**A**), CYP1A2 (**B**) and GAPDH (**C**) as control. UT, untreated cells kept in culture for the same time as treated cells, (−) absence of inducer. Relative RNA amounts were determined by densitometry, and normalized to GAPDH mRNAs. Autoradiography are representative of three separate experiments.

of the subsequent induced CYP1A transcription. On the contrary, the TBZ protective influence against cytokine repression of induced CYP1A-related activities, could only be explained by the enhancement of P450 catalytic activity, although possible direct stimulation of P450 enzymes has not been yet reported for this kind of compound.

Finally, we demonstrated that carcinogen-metabolizing P4501A subfamily expression is differentially modulated by cytokines. This phenomenon might have relevance to the process of chemical carcinogenesis and could contribute to interindividual variations in cancer susceptibility, through the degree of inflammation. Moreover, an increasing number of clinical trials include therapeutical use of IL-2 and IFN-γ for the treatment of pathologic conditions, such as viral hepatitis (33). Since the combination of cytokines has been shown to produce synergistic effects, it is now necessary to understand their specific mechanisms of repression, in order to prevent therapeutic side effects.

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