	品名, 規格単位	適応, 用法・用量	警告, 禁忌, 副作用等
抗B型肝炎ウイルス薬(核酸アナログ製剤)		スを有する等)には1日1回1mgが推奨	(脂肪肝)〕(その他(5%以上)) 頭痛, 血中Bil・血中アミラーゼ・リパーゼ・血中ブドウ糖・血中乳酸増加, BUN上昇, 白血球数減少, (ヌクレオシド類縁体未治療患者のみ)下痢, (ラミブジン不応患者のみ)悪心, 倦怠感, 鼻咽頭炎, AST・ALT上昇, 尿潜血・尿中白血球陽性, 好酸球数増加 (半減期) 97時間(0.5mg反復) (排泄) 主に腎 (妊婦) 妊娠する可能性:避妊を指導 (授乳婦) 授乳中止
		臨床情報	(処方のPoint) 内服薬. ラミブジンと比較してB型肝炎に対する長期投与に伴う耐性ウイルスの出現率が低いのが特徴. (腎機能) 腎不全: B (Ccrに応じ投与間隔延長). 透析時: B (通常用量を週1回投与) (調剤・薬学管理のPoint) 定期的な腎機能モニタリングと用量調整が必要 (指導) 継続投与・空腹時投与の必要性
	ラミブジン(lamivudine), 3TC		(劇)
	ゼフィックス (GSK) (錠) 100mg ¥622.2 粉砕(○)	B型肝炎ウイルスの増殖を伴い肝機能の異常が確認されたB型慢性肝疾患におけるB型肝炎ウイルスの増殖抑制 ➡ 1日1回100mg	(警) 投与終了後, ウイルス再増殖に伴い肝機能の悪化もしくは肝炎の重症化が認められることがあるため, 投与終了後少なくとも4カ月間は原則として2週間毎に患者の臨床症状と臨床検査値(HBV DNA, ALT及び必要に応じT-Bil)を観察. 特に免疫応答の強い患者・非代償性肝疾患では, 投与終了後に肝炎が重症化することがあり, 投与終了後の経過観察をより慎重に行う必要あり (副) (重大) 血小板減少, 横紋筋融解症, 類薬〔重篤な血液障害(赤芽球癆, 汎血球・白血球・好中球・血小板減少, 貧血), 膵炎, 乳酸アシドーシス及び脂肪沈着による重度の肝腫大(脂肪肝), 横紋筋融解症, ニューロパシー, 錯乱, 痙攣, 心不全〕(その他(5%以上)) 肝機能障害・肝機能悪化 (併注) ST合剤 (半減期) β8.2時間(100mg) (授乳婦) 授乳回避
		臨床情報	(処方のPoint) 内服薬. B型肝炎ウイルスに対する強力な核酸アナログ製剤. 長期投与に伴う耐性ウイルス出現と, 中止後の肝炎悪化が問題となる (腎機能) 腎不全時: B (Ccrに応じ減量及び投与間隔延長). 透析時: B (Ccrに応じて減量) (調剤・薬学管理のPoint) 定期的な腎機能モニタリングと用量調整が必要 (指導) 継続投与の必要性
肝機能改善薬	**ウルソデオキシコール酸**		
	ウルソ	胆道疾患治療薬・膵臓疾患治療薬➡540頁参照	

品名，規格単位	適応，用法・用量	警告，禁忌，副作用等
グリチルリチン・グリシン・システイン配合剤(glycyrrhizin・glycine・cysteine combined)		
強力ネオミノファーゲンシー 注〔静注〕5mL ¥64，20mL ¥130(後60) （ミノファーゲン） 新 注〔静注・シリンジ〕20mL ¥230(後160～206)，40mL ¥371(後272～302) （ミノファーゲン＝エーザイ） 09年5月収載 いずれも1mL中グリチルリチン酸2mg，グリシン20mg，L-システイン塩酸塩1mg含有	①湿疹・皮膚炎，蕁麻疹，皮膚瘙痒症，薬疹・中毒疹，口内炎，小児ストロフルス，フリクテン ➡1日1回5～20mLを静注 ②慢性肝疾患における肝機能異常の改善 ➡1日1回40～60mLを静注，点滴静注（1日最高）100mL	禁 アルドステロン症，ミオパシー，低K血症 副 重大 ショック，アナフィラキシーショック，アナフィラキシー様症状，偽アルドステロン症 併注 ループ利尿薬，サイアザイド系及びその類似降圧利尿薬，モキシフロキサシン
	他製品 キョウミノチン，グリファーゲン，グリファーゲンC，グルコリンS，ケベラS，ニチファーゲン，ネオファーゲン，ネオファーゲンC，ノイファーゲン，レミゲン，レミゲンM 後発品 アスファーゲン，アミファーゲンP，グリベルチン，チスファーゲン，ヒシファーゲンC，ミノフィット	
	臨床情報 処方のPoint 静注製剤．ウイルス量を減少させる効果はないもののALT値を低下させる抗炎症療法としてC型肝炎では特に有用 腎機能 腎不全時：A，透析時：A 調剤・薬学管理のPoint 定期的な電解質等のモニタリング（低K血症，血圧上昇，Na・体液の貯留，浮腫，体重増加等の偽アルドステロン症の恐れ）が必要	
グルクロノラクトン(glucuronolactone)		
グロンサン （中外） 末 1g ¥10.9	高Bil血症（閉塞性黄疸を除く）における肝機能の改善，蕁麻疹，湿疹，中毒疹，妊娠悪阻，妊娠中毒 ➡1回0.3～1gを1日3回	
グルクロン酸ナトリウム水和物(sodium glucuronate hydrate)		
グロンサン （中外） 注（10％）200mg/2mL ¥61，（25％）500mg/2mL ¥61	高Bil血症（閉塞性黄疸を除く）における肝機能の改善，妊娠悪阻，妊娠中毒 ➡1日50～500mgを皮下注，筋注，静注，又は1日1000mgを静注	
ジクロロ酢酸ジイソプロピルアミン(diisopropylamine dichloroacetate)		
リバオール （第一三共） 散（10％）100mg/g ¥25.2 錠 20mg ¥6.2	慢性肝疾患における肝機能の改善 ➡1日20～60mgを2～3回に分服	
タウリン（別名：アミノエチルスルホン酸）(taurine)		
タウリン （大正製薬＝大正富山）	高Bil血症（閉塞性黄疸を除く）における肝機能の改善，うっ血性心	半減期 2時間（本剤2g）

肝機能改善薬

29 肝疾患治療薬

	品名,規格単位	適応,用法・用量	警告,禁忌,副作用等
肝機能改善薬	散 (98%)1g¥10.6	不全 ➡ 1回本剤1gを1日3回,食後 (うっ血性心不全) 強心利尿薬で十分な効果が認められないときに併用	
	チオプロニン(tiopronin)		
	チオラ (マイラン) 錠 100mg¥11.6 粉砕	①慢性肝疾患における肝機能の改善 ➡ 1回100mgを1日3回 ②初期老人性皮質白内障 ➡ 1回100～200mgを1日1～2回 ③水銀中毒時の水銀排泄増加 ➡ 1回100～200mgを1日3回 ④シスチン尿症 ➡ 1回100mgから開始し,1日4回,食後・就寝前 (1回最高) 500mg(1日2g) 小児:1日100mgから開始し,(1日最高) 40mg/kg,但し1日2gを超えない	(副) (重大) 中毒性表皮壊死症,天疱瘡様症状,黄疸,無顆粒球症,間質性肺炎,ネフローゼ症候群,慢性関節リウマチへの大量投与(重症筋無力症,多発性筋炎) (その他(5%以上)) (適応④のみ)消化器障害,瘙痒感,風邪症候群 (半減期) 1.2時間(400mg) (授乳婦) 授乳中止
		臨床情報 (調剤・薬学管理のPoint) (モニタ) 定期的な肝機能検査(特に投与後2,4,6週の検査),消化器症状,発熱,倦怠感等(黄疸等の重篤な副作用の恐れ)	
	プロトポルフィリンニナトリウム(protoporphyrin disodium)		
	プロルモン (田辺三菱=田辺販売) 錠 20mg▼ ¥7.8(後6.1～7.8)	慢性肝疾患における肝機能の改善 ➡ 1回20～40mgを1日3回	(併注) メトキサレン
		(後発品) プロトポルト,プロルモン,ポルフラジン,レバスダン	
	ポリエンホスファチジルコリン(polyenephosphatidyl choline)		
	EPL (アルフレッサファーマ) 力 250mg▼ ¥9.2(後6.2～9.2)	慢性肝疾患における肝機能の改善,脂肪肝,高脂質血症 ➡ 1回500mgを1日3回	
		(後発品) EPL,プロビーン	
肝機能改善薬(肝臓加水分解物)	**肝臓加水分解物(liver hydrolysate)**		
	レバイデン (東邦新薬) 顆〔G〕(20%)200mg/g ¥9.8 錠 100mg¥6.1	慢性肝疾患における肝機能の改善 ➡ 1回200mgを1日3回	(禁) 肝性昏睡
		(後発品) ゴスペール・レバー,レナルチン,レバラミン	

分類	品名,規格単位	適応,用法・用量	警告,禁忌,副作用等
肝機能改善薬肝臓加水分解物	**肝臓加水分解物配合剤(liver hydrolysate combined)**		
	プロヘパール (科研) 錠(肝臓加水分解物,塩酸システイン,重酒石酸コリン,イノシトール,シアノコバラミン)1錠¥7.6	慢性肝疾患における肝機能の改善 ➡1回1～2錠を1日3回	禁 肝性昏睡
		臨床情報 腎機能 腎不全時:A,透析時:A 調剤・薬学管理のPoint アンモニア血症を助長することがあるため,肝性昏睡には投与しない	
肝機能改善薬(肝臓抽出製剤)	**肝臓エキス・フラビンアデニンジヌクレオチド(liver extract・flavin adenine dinucleotide)**		
	アデラビン9号 (三和化学) 注 1mL¥169(後60～91),2mL¥289(後79～106) いずれも1mL中肝臓エキス15μL,フラビンアデニンジヌクレオチド10mg	慢性肝疾患における肝機能の改善,次の疾患(湿疹・皮膚炎群,口唇炎・口角炎・口内炎,びまん性表層角膜炎)のうちビタミンB_2の欠乏又は代謝障害が関与すると推定される場合,ビタミンB_2の需要が増大し,食事からの摂取が不十分な際の補給(消耗性疾患,妊産婦,授乳婦等) ➡1日1～2mLを1～2回に分けて皮下注,筋注,静注	副 重大 ショック
		後発品 アスルダム,アセラート,エフエーミック,ヒノマリン,ベマカスト,リバレス,レバサルト	
		臨床情報 腎機能 腎不全時:A,透析時:B(投与間隔延長) 調剤・薬学管理のPoint 指導 尿を黄変させる可能性がある	
アレルギー用薬	**グリチルリチン酸・DL-メチオニン配合剤(glycyrrhizic acid・DL-methionine combined)**		
	グリチロン (ミノファーゲン) 錠(グリチルリチン酸25mg,グリシン25mg,DL-メチオニン25mg)1錠¥6.2 粉砕○	慢性肝疾患における肝機能異常の改善.湿疹・皮膚炎,小児ストロフルス,円形脱毛症,口内炎 ➡1回2～3錠(小児 1錠)を1日3回,食後	禁 アルドステロン症,ミオパシー,低K血症,血清アンモニウム値上昇傾向にある末期肝硬変症 副 重大 偽アルドステロン症 併注 ループ利尿薬,サイアザイド系・類似利尿薬,モキシフロキサシン
		臨床情報 腎機能 腎不全時:A,透析時:A 調剤・薬学管理のPoint モニタ 血圧,血清K,尿量,体重,CPK,甘草を含有する製剤の併用 指導 頭痛,むくみ,脱力感,筋肉痛	
肝蛋白代謝改善薬	**マロチラート(malotilate)**		
	カンテック (第一三共) 錠 200mg¥111.9 粉砕(×)	肝硬変(代償性)における肝機能の改善 ➡1日600mgを3回に分服	禁 黄疸,腹水,肝性脳症 副 重大 黄疸,腹水,AST・ALT・Bilの高度上昇等の重篤な肝障害 その他(5%以上) 食欲不振・嘔吐等の消化器症状,Bil上昇等の肝機能異常

▼…後発品 ●…剤形が見出し製品と異なるものあり ■…適応が見出し製品と異なるものあり

29 肝疾患治療薬

	品名,規格単位	適応,用法・用量	警告,禁忌,副作用等
		(半減期)(未変)6.7時間,(活性)8.4時間(200mg)(授乳婦)授乳回避	
免疫賦活薬	**プロパゲルマニウム**(propagermanium)		
	セロシオン (三和化学=アステラス) (カ)10mg ¥203.5 粉砕(○)	HBe抗原陽性B型慢性肝炎におけるウイルスマーカーの改善 ➡1日30mgを3回に分服,食後.開始16週目にウイルスマーカー(HBe抗原等)を含めた臨床検査を実施し,ウイルスマーカーの改善がみられなかった場合には他の療法を考慮	(警)慢性肝炎が急性増悪することがあり,死亡例の報告あり (禁)黄疸,肝硬変又はその疑い (副)(重大)B型慢性肝炎の急性増悪 (半減期)2.4時間(15mg)(授乳婦)授乳中止(高齢者)1日20mgから開始するなど慎重に投与
		臨床情報 (調剤・薬学管理のPoint) B型慢性肝炎重症化の可能性を有するため,黄疸,肝硬変,肝硬変疑いには投与しない	
高アンモニア血症改善薬	**L-アルギニンL-グルタミン酸塩水和物**(L-arginine L-glutamate hydrate)		
	アルギメート (味の素=味の素ファルマ) (注)(10%)20g/200mL ¥625	高アンモニア血症 ➡1日2～20gを1～数回に分けて点滴静注	(併注)全身麻酔薬,イソニアジド,イオン交換樹脂,サイアザイド系利尿薬(授乳婦)授乳回避
	L-グルタミン酸ナトリウム水和物(sodium L-glutamate hydrate)		
	アンコーマ (東亜薬工=鳥居) (注)〔点滴静注〕4g/20mL ¥225	高アンモニア血症 ➡4～8gを5%(ブ)に混合したもの約100mLを点滴静注	
	ラクチトール水和物(lactitol hydrate)		
	ポルトラック (日本新薬) (末)1g ¥7.5	非代償性肝硬変に伴う高アンモニア血症 ➡1日量18～36gを3回に分けて,用時水に溶解後経口投与.下痢が引き起こされることがあるので初回投与量は1日量18gとして漸増,便通状態として1日2～3回程度の軟便がみられる量を投与(1日最高)36g.水様便が現れた場合には減量又は一時中止	(禁)ガラクトース血症 (併注)αグルコシダーゼ阻害薬
		臨床情報 (腎機能)腎不全時:A.透析時:A (調剤・薬学管理のPoint) ガラクトース血症の患者には投与しない	
	ラクツロース(lactulose)		
	モニラック (中外) (散)1g ¥7.7 (シ)(65%)650mg/mL ¥6.4((後)4.8)	①高アンモニア血症に伴う精神神経障害・手指振戦・脳波異常の改善 ➡(モニラック(散))1日19.5～39gを3回に分服.(ラクツロー	(禁)ガラクトース血症 (副)(その他(5%以上))(モニラック,ラクツロース)下痢 (併注)αグルコシダーゼ阻害薬

	品名，規格単位	適応，用法・用量	警告，禁忌，副作用等
高アンモニア血症改善薬	**ラクツロース** （興和＝興和創薬） 散〔P〕1g ¥10 シ（60％）600mg/mL ¥6.6 **カロリール** （佐藤製薬） 内用ゼリー（40.496％）1g▼ ¥4.1（後4.1）	ス）1日18～36gを用時溶解し（シは1日本剤30～60mL），2～3回に分服．（モニラック シ）1日本剤30～60mLを3回に分服 内用ゼリー 本剤48.1～96.2gを3回に分服 ②（ラクツロース除く）産婦人科術後の排ガス・排便の促進 ➡散 1日19.5～39gを2回に分服，朝夕 シ 1日本剤30～60mLを2回に分服，朝夕 内用ゼリー 本剤48.1～96.2gを2回に分服，朝夕 ③（ラクツロース，カロリール除く）小児における便秘の改善 ➡散 1日0.33～1.3g/kgを3回に分服． シ 1日本剤0.5～2mL/kgを3回に分服 後発品 カロリール，ピアーレ，ラグノス，リフォロース 臨床情報 腎機能（モニラック，ラクツロース）腎不全時：A，透析時：A 調剤・薬学管理のPoint ガラクトース血症の患者には投与しない	
肝炎ワクチン	乾燥組織培養不活化A型肝炎ワクチン		
	エイムゲン	予防接種用薬➡1258頁参照	
	組換え沈降B型肝炎ワクチン		
	ビームゲン **ヘプタバックス－Ⅱ** **沈降B型肝炎ワクチン**	予防接種用薬➡1258頁参照	
	小柴胡湯		
	小柴胡湯	漢方薬➡1339頁参照	

29 肝疾患治療薬

▼…後発品 ●…剤形が見出し製品と異なるものあり ■…適応が見出し製品と異なるものあり

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ORIGINAL ARTICLE

Natural killer cell is a major producer of interferon γ that is critical for the IL-12-induced anti-tumor effect in mice

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Abstract Although the anti-tumor effect of IL-12 is mediated mostly by IFNγ, which cell types most efficiently produce IFNγ and therefore initiate or promote the anti-tumor effect of IL-12 has not been clearly determined. In the present study, we demonstrated hydrodynamic injection of the IL-12 gene led to prolonged IFNγ production, NK-cell activation and complete inhibition of liver metastasis of CT-26 colon cancer cells in wild-type mice, but not in IFNγ knockout mice. NK cells expressed higher levels of STAT4 and upon IL-12 administration displayed stronger STAT4 phosphorylation and IFNγ production than non-NK cells. Adoptive transfer of wild-type NK cells into IFNγ knockout mice restored IL-12-induced IFNγ production, NK-cell activation and anti-tumor effect, whereas transfer of the same number of wild-type non-NK cells did not. In conclusion, NK cells are predominant producers of IFNγ that is critical for IL-12 anti-tumor therapy.

Keywords IFNγ · Innate immunity · Liver tumor · IL-12 · NK

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Introduction

IL-12 is a 70-kDa heterodimer protein, composed of p35 and p40 subunits, mainly produced by antigen-presenting cells. IL-12 was originally found as a "natural killer-stimulating factor" and a "cytotoxic lymphocyte maturation factor" [1, 2]. IL-12 has multi-potent effects, inducing a Th1 response, enhancing the CD8 T-cell response, activating natural killer cells and inducing production of IFNγ [3, 4]. Therapeutic use of IL-12, either using its recombinant protein or gene, can induce an efficient anti-tumor effect on primary or metastatic tumors in various murine models and humans [5, 6].

Research has shown that IL-12 mediates anti-tumor effects in a variety of ways. They include anti-proliferative effects, anti-angiogenic effects [7, 8] and cytotoxic effects of effector lymphocytes. A variety of effector cells has been reported to be required for IL-12-mediated anti-tumor effects: they include CD8 T cells [9], NKT cells [10], CD4 T cells [11] and NK cells [12]. The relative contribution of these cells may differ among IL-12 doses and types of tumor models [13]. Endogenous IFNγ production is required for most, if not all, of the anti-tumor effects of IL-12 administration [14, 15]. IL-12 stimulates a variety of immune cells, such as T cells [16], B cells [17] and NK cells [18], to produce IFNγ. However, which cell types are most critical for producing IFNγ during IL-12 therapy is not clearly known.

In the present study, we used a murine model of liver metastasis of CT-26 colon cancer cells and found that NK cells highly expressed the IL-12 signaling molecule STAT4 and most efficiently produced IFNγ. IFNγ was essential for the anti-tumor effect of IL-12, and NK-cell production of IFNγ sufficed to produce the full-blown anti-tumor effects. These results demonstrated that NK cells

serve not only as an effector but also as an important mediator producing IFNγ that is critical for the anti-tumor effects of IL-12.

Materials and methods

Mice

Specific pathogen-free female Balb/c mice were purchased from Clea Japan, Inc (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice with a Balb/c background were purchased from Taconic (Germantown, NY). IFNγ knockout (GKO) mice with a Balb/c background were kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo). All mice used were at the age of 6 to 10 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care, and the study protocol complied with the institution's guidelines.

Tumor models

Intra-splenic injection of tumor cells was used to establish micro-disseminated liver tumors in mice [19]. CT-26 colon cancer cells originating from Balb/c mice were maintained in RPMI1620 supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 200 μl of PBS and injected into the spleen.

Injection of naked plasmid DNA

A plasmid coding the murine IL-12 gene, pCMV-IL-12, was generously provided by Dr. M Watanabe (Laboratory of Experimental Immunology, Division of Basic Sciences, National Cancer Institute-Frederick Cancer Research and Development Center) [20]. Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany,) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed as previously described [21]. In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein, using a syringe with a 26-gauge needle. DNA injection was completed within 5 to 8 s.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels of serum IL-12 p70, IFNγ (BD Biosciences-Pharmingen, San Diego, CA), IFNγ-inducible protein 10 (IP-10) and monokine induced by IFNγ (MIG) (R&D Systems, Inc, Minneapolis, MN) were measured using commercially available ELISA kits in accordance with the manufacturer's instructions.

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described. The NK activity of mononuclear cells was assessed by a standard 4-h ^{51}Cr-releasing assay using Yac1 cells as targets. In some experiments, mononuclear cells were separated into DX5$^+$ cells (NK cells) and DX5$^-$ cells (non-NK cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated NK-cell population was found to be greater than 90% by FACS analysis.

Flow cytometric analysis

Liver mononuclear cells were isolated 2 days after pCMV-IL-12 injection. Cytokine secretion was then blocked by the addition of brefeldin A for 4 h. Next, liver mononuclear cells were stained with FITC-conjugated anti-TCRβ antibody and biotin-conjugated anti-CD49b antibody (DX5), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained with PE-conjugated anti-INFγ antibody or corresponding isotype controls. Analysis was performed using a FACSCalibur (Becton Dickinson), with the resulting data analyzed using the CELLQuest program (Becton Dickinson). NK cells were identified as DX5$^+$/TCRβ$^-$ lymphocytes, NKT cells as DX5$^+$/TCRβ$^+$ lymphocytes and T cells as DX5$^-$/TCRβ$^+$ lymphocytes.

Adoptive transfer

For adoptive transfer experiments, GKO mice were injected intravenously 1 day before plasmid DNA injection with 2.0×10^8 whole mononuclear cells or 4.0×10^6 NK cells, or non-NK cells or whole mononuclear cells, all of which had been harvested from wild-type mice that can produce IFNγ.

Western blotting

Mouse recombinant IL-12 was purchased from R&D Systems, Inc (Minneapolis, MN). Mononuclear cells were treated with or without IL-12. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μg of protein was separated by SDS-PAGE and transferred to the PVDF membrane. The membrane was stained with anti-STAT4 antibody (BD biosciences),

anti-phospho-specific STAT4 (pY693) antibody (BD biosciences), anti-STAT1 antibody (Cell Signaling), anti-phospho-specific STAT1 antibody (Cell Signaling) and visualized by chemiluminescence.

NK-cell depletion

For depletion of NK cells in vivo, anti-asialoGM1 antibody (WAKO, Osaka, Japan) was intraperitoneally administered. We determined the appropriate dosing to be 500 μg/mouse (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. The percentage of $DX5^{+}/TCR\beta^{-}$ cells (NK cells) is 12.6 ± 2.4% in IgG-injected liver, whereas it decreased to 0.76 ± 0.04% one day after anti-asialo GM1 antibody injection ($N = 3$/group). This effect remained at least 3 days after anti-asialo GM1 antibody injection. NKT cells were less affected than NK cells, because 90% of $DX5^{+}/TCR\beta^{+}$ cells (NKT cells) still remained in the liver after the treatment. Anti-asialoGM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Histology

The formalin-fixed livers were paraffin-embedded, and liver sections were analyzed by hematoxylin-eosin staining. Acetone-fixed fresh frozen liver sections were immunostained with anti-mouse CD4 (H123.19), anti-mouse CD8α (53-6.7) or anti-CD31 (390) monoclonal antibody (all from BD Biosciences), using a VECSTAIN ABC kit (Vector Laboratories, Burlingame, California, USA).

Statistics

Data are represented as mean ± SD. Comparisons between groups were analyzed by unpaired t-test with Welch's correction. $p < 0.05$ was considered statistically significant.

Results

Hydrodynamic injection of IL-12-expressing plasmid led to prolonged production of IFNγ

Hydrodynamics-based gene delivery into mice establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes. Serial measurement of serum IL-12 demonstrated that pCMV-IL-12 injection led to substantial IL-12 production on day 1. The levels of serum IL-12 then rapidly declined (Fig. 1a). We also measured IFNγ production in serum, since IL-12 is known to activate IFNγ production. pCMV-IL-12 and, to a lesser extent, pCMV injection increased serum IFNγ on day 1. In contrast to the pCMV injection group, high levels of serum IFNγ were maintained at later time points in the pCMV-IL-12 injection group (Fig. 1a). Thus, hydrodynamic injection of pCMV-IL-12 led to prolonged production of IFNγ. Transient IFNγ production followed by control plasmid may be an indirect effect of liver injury caused by bolus injection of saline or DNA injection.

IL-12 therapy induced NK activation and anti-metastatic effects, both of which are critically dependent on IFNγ

To examine the biological effects of the produced IL-12, we evaluated the NK activity of mononuclear cells from the liver. pCMV-IL-12 injection, but not control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells (Fig. 1b). When GKO mice were injected with pCMV-IL-12 or pCMV, the hepatic mononuclear cells did not display any lytic ability to Yac1 cells, suggesting that IL-12-mediated NK-cell activation required IFNγ.

To examine the anti-metastatic effect of IL-12, pCMV-IL-12 or pCMV was injected into wild-type mice 2 days after intrasplenic injection of CT-26 cells. At 14 days after tumor injection, the mice were killed for evaluation of liver tumor (Fig. 1c). While pCMV-injected mice displayed huge liver tumors, pCMV-IL-12-injected mice did not show any macroscopic or microscopic tumor (Fig. 1d). Liver weight was significantly higher in pCMV-injected mice than pCMV-IL-12-injected mice, reflecting liver tumor formation. To examine the involvement of IFNγ in the IL-12-induced anti-tumor effect, we injected pCMV or pCMV-IL-12 into GKO mice 2 days after CT-26 injection. At 14 days after CT-26 injection, both groups showed similar degrees of tumor formation and there was no significant difference in liver weight between the two. This indicated that IL-12-induced anti-metastatic effect was strictly dependent on IFNγ.

NK cells were the most potent producer of IFNγ during IL-12 therapy

To evaluate which cell types most efficiently produced IFNγ, we isolated hepatic mononuclear cells from mice 2 days after plasmid injection and then stained cell surface TCRβ and DX5 as well as intracellular IFNγ (Fig. 2). $TCR\beta^{-}/DX5^{+}$ NK cells, $TCR\beta^{+}/DX5^{+}$ NKT cells and $TCR\beta^{+}/DX5^{-}$ T cells from pCMV-IL-12-injected mice showed significant levels of IFNγ production compared

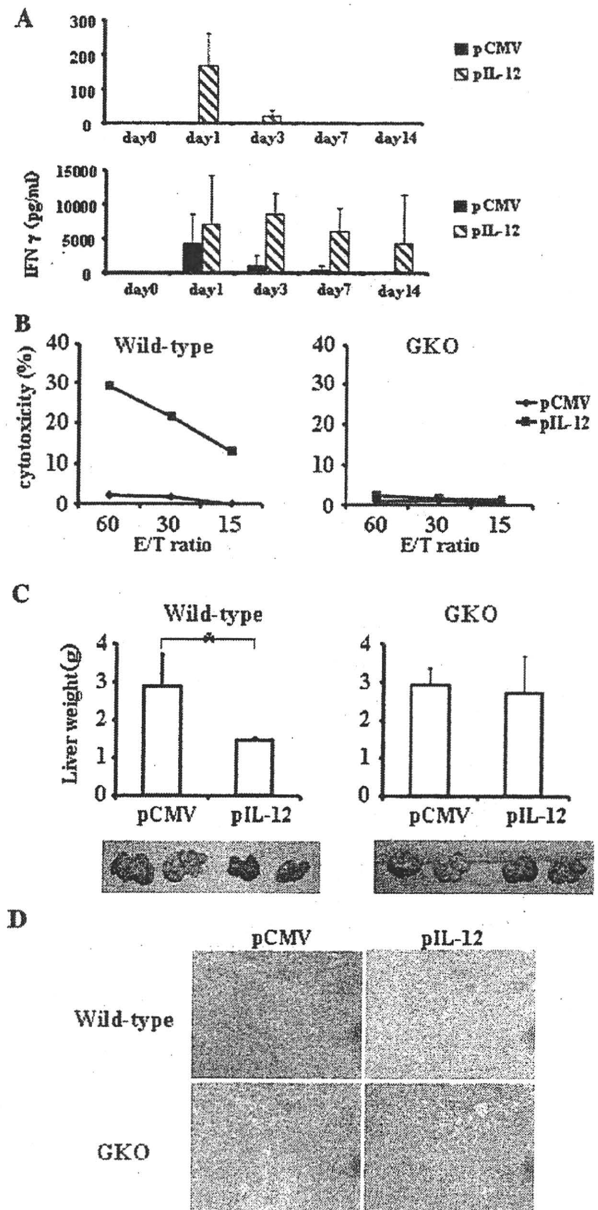

Fig. 1 Effects of hydrodynamic injection of IL-12-encoding plasmid. **a** Wild-type mice were hydrodynamically injected with either pCMV-IL-12 (*hatched bars*) or pCMV (*closed bars*) and bled at the indicated time points to measure the levels of serum IL-12 and IFNγ. Results are indicated as mean and SD ($n = 6$/group). **b** NK-cell activation after IL-12 administration. Hepatic mononuclear cells were isolated from wild-type mice (*left*) or GKO mice (*right*) which had been injected with pCMV-IL-12 (*closed squares*) or pCMV (*closed diamonds*) 4 days earlier. Yac1 lytic ability was measured by a standard ^{51}Cr-release assay at the indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown. **c** and **d** Anti-metastatic effects of IL-12 therapy. Wild-type mice (*left*) or GKO mice (*right*) were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. At 14 days after the plasmid injection, the mice were killed to examine liver tumor development. **c** Data are indicated as mean and SD of the liver weight at the top ($n = 6$/group) and a representative picture of the liver in each group is shown at the bottom. *$p < 0.001$. **d** Representative histology of liver sections

with those from naive mice or pCMV-injected mice. The levels of IFNγ production were highest in NK cells among those cells. Even at a later time point, 7 days after plasmid injection, NK cells were found to produce the highest levels of IFNγ (data not shown).

IL-12-induced STAT4 signaling and IFNγ production increased in NK cells

IL-12 activates Janus kinases Tyk2 and Jak2, STAT4 as well as other STATs. To examine the activation of STAT1 and STAT4, we isolated splenocytes from wild-type mice and GKO mice and stimulated them with IL-12 and/or IFNγ in the presence or absence of anti-IFNγ Ab (Fig. 3a). IL-12 led to phosphorylation of both STAT1 and STAT4 in wild-type splenocytes. In contrast, the same treatment led to phosphorylation of STAT4, but not of STAT1, in GKO splenocytes. Addition of IFNγ restored STAT1 phosphorylation in GKO splenocytes. Furthermore, adding anti-IFNγ inhibited STAT1 phosphorylation in wild-type cells. These findings demonstrated that phosphorylation of STAT4 is a direct effect of IL-12 but phosphorylation of STAT1 is indirect, via an autocrine or paracrine IFNγ-dependent manner.

To examine STAT1 and STAT4 activation and IFNγ production in NK cells and non-NK cells, we prepared whole mononuclear cells as well as NK and non-NK populations from wild-type spleens and stimulated the cells with IL-12 (Fig. 3b). NK cells expressed higher levels of STAT4 than non-NK cells. Upon IL-12 treatment, STAT4 was rapidly phosphorylated in NK cells, but to a lesser extent in non-NK cells. In contrast, NK cells expressed lesser levels of STAT1 than non-NK cells. STAT1 was similarly phosphorylated in NK cells and non-NK cells upon IL-12 treatment. Both NK cells and non-NK cells produced significant levels of IFNγ, but the levels were much higher in NK cells than non-NK cells (Fig. 3c). These results indicated that compared with non-NK cells, NK cells possessed higher levels of STAT4, a direct signaling molecule of IL-12, and produced higher levels of IFNγ than non-NK cells.

NK cells were sufficient for IL-12-mediated anti-tumor effects

The above observation indicated that NK cells are a predominant producer of IFNγ, which was critical for the IL-12-induced anti-tumor effects. To examine whether NK

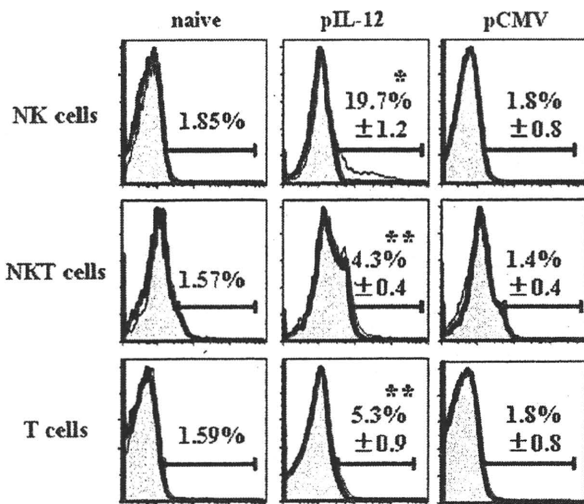

Fig. 2 IFNγ expression of mononuclear cells after IL-12 administration. Wild-type mice were injected with pCMV-IL-12 or pCMV, or were untreated (naive). Mononuclear cells were isolated from the liver 2 days after plasmid injection and stained with anti-TCRβ mAb, anti-DX5 mAb and anti-IFNγ mAb. *Closed histograms* show the IFNγ expression in the gated populations (TCRβ⁻/DX5⁺ cells for NK cells, TCRβ⁺/DX5⁺ cells for NKT cells and TCRβ⁺/DX5⁻ cells for T cells). Isotype control stainings are shown by *open histograms*. Numbers in histograms represent averages ± SD of percentages of positive cells ($n = 3$ mice/group). $^{*}p < 0.0001$ vs. mock in NK populations. $^{**}p < 0.05$ vs. mock in each population

cells are sufficient for the anti-metastatic effects of IL-12, we examined the anti-metastatic effect in Rag2 KO mice which lack T cells, B cells and NKT cells. pCMV-IL-12 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in Rag2 KO mice higher than in wild-type mice (Fig. 4a). To examine whether NK cells are sufficient for IL-12-mediated rejection of hepatic metastasis, we injected pCMV-IL-12 or pCMV into mice that had been intra-splenically injected with CT-26 cells 2 days earlier. Serum IFNγ levels of Rag2 KO mice were about 4 times higher than those of wild-type mice (Fig. 4b). pCMV-IL-12 completely suppressed hepatic metastasis in Rag2 KO mice (Fig. 4c).

Adoptive transfer of wild-type NK cells into GKO mice restored the anti-tumor effects of IL-12

Since NK cells were sufficient for producing IL-12-induced anti-tumor effects, we postulated that their production of IFNγ may play an important role in these effects. To test this, we performed adoptive transfer experiments with GKO mice. First, whole mononuclear cells isolated from the spleens of wild-type mice (2.0×10^8 cells) were adoptively transferred to GKO mice 1 day before plasmid injection. pCMV-IL-12 injection increased Yac1 lytic activity of hepatic mononuclear cells in the adoptively

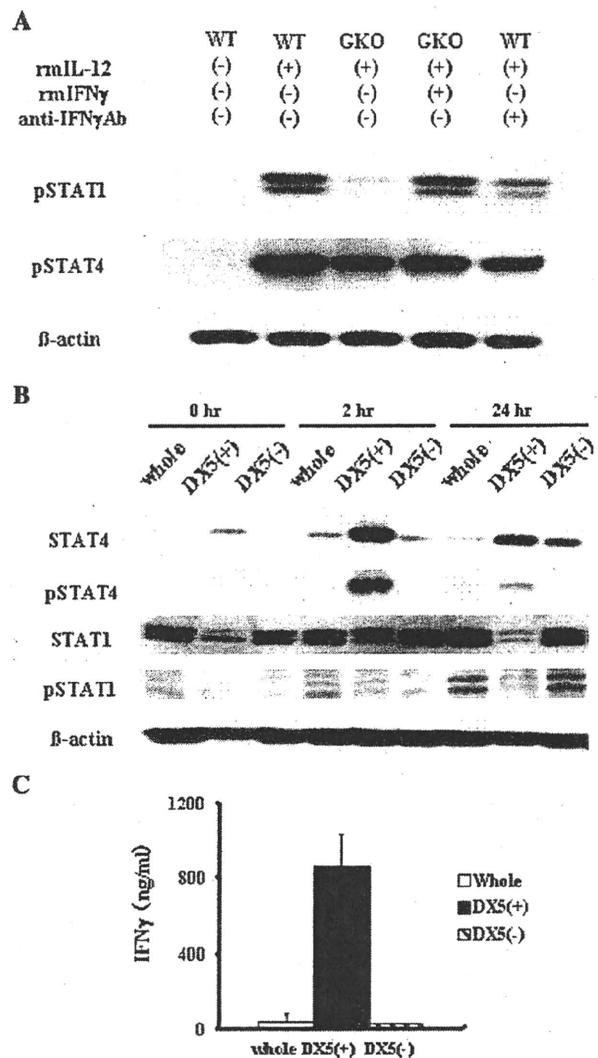

Fig. 3 STAT signaling and IFNγ production of mononuclear cells in vitro treated with IL-12. **a** STAT1 and STAT4 activation of splenocytes in vitro treated with IL-12. Splenocytes were isolated from wild-type mice or GKO mice and treated with or without recombinant IL-12 (20 ng/mL) in the presence or absence of recombinant IFNγ (500 ng/mL) or anti- IFNγ antibody (20 μg/mL) for 24 h. Cellular lysates were analyzed by Western blot for the expression of phospho-STAT1, phospho-STAT4 and β-actin. **b** and **c** STATs expression and signaling of NK cells and non-NK cells. Splenocytes were isolated from wild-type mice. Whole splenocytes were further purified into DX5⁺ cells and DX5⁻ cells. Each cell population was cultured with recombinant IL-12 (20 ng/mL) for the indicated times. **b** The cells were lysed to examine expression of whole STAT and phospho-STAT by Western blot. **c** The levels of IFNγ in the culture supernatant at 24 h were determined by ELISA. Data are expressed as mean and SD ($n = 3$)

transferred group, but not in the untreated group (Fig. 5a). pCMV-IL-12 induced significant increase in serum IFNγ levels 4 days after plasmid injection in the adoptive transferred group, but not in the other groups (Fig. 5b). The

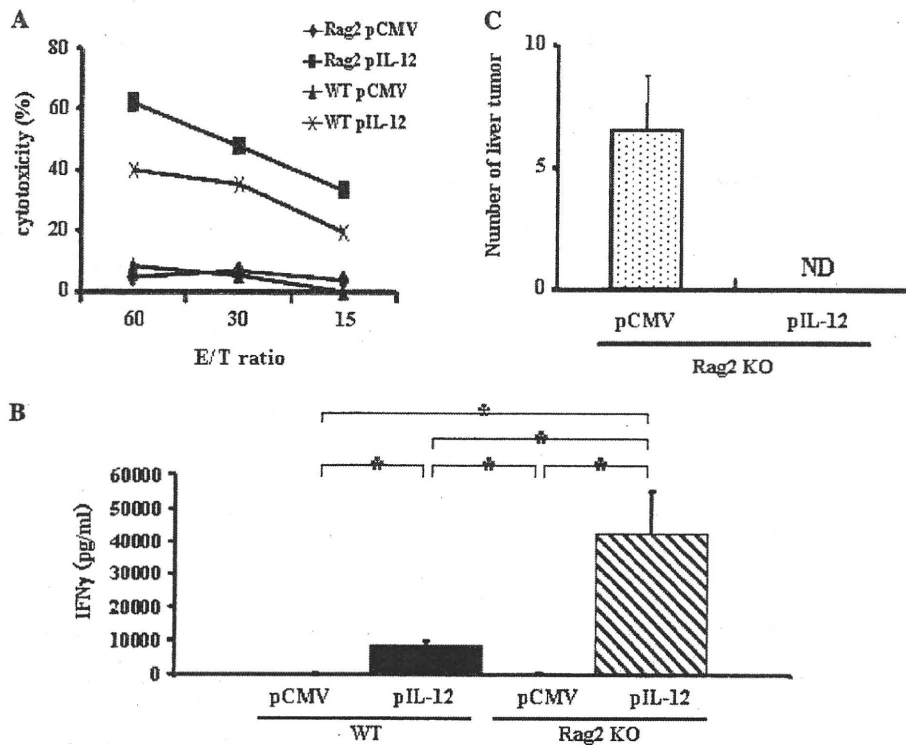

Fig. 4 Anti-tumor effects of IL-12 in Rag2 KO mice. Serum IFNγ levels and NK-cell activation. Wild-type or Rag2 KO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV and killed at 4 days. **a** Yac1 lytic ability of hepatic mononuclear cells was determined by Cr releasing assay as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFNγ were determined by ELISA. Data are expressed as mean and SD ($n = 7$/group). *$p < 0.0001$. **c** Anti-metastatic effect. Rag2 KO mice were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Fourteen days after plasmid injection, mice were killed to examine tumor development in the liver. The numbers of hepatic tumors in each group are expressed as mean and SD ($n = 7$/group). *ND* not detectable

anti-metastatic effect of IL-12 was restored in GKO mice when whole mononuclear cells from wild-type mice were adoptively transferred (Fig. 5c).

To evaluate the contribution of IFNγ production from each subset of mononuclear cells to the anti-metastatic effect of IL-12, we adoptively transferred the same number of whole mononuclear cells, NK cells or non-NK cells from wild-type mice (4.0×10^6 cells) 1 day before pCMV-IL-12 injection and analyzed liver tumor formation. Only in the NK-cell-transferred group, pCMV-IL-12 injection induced NK cytolytic ability in the liver and IFNγ elevation in serum 4 days after plasmid injection, but not in the other groups (Fig. 5d, e). No liver tumor formed in the NK-cell-transferred group. In contrast, livers in other groups had massive tumors, and the liver weights were significantly heavier than those in the NK-cell-transferred group (Fig. 5f). These results clearly demonstrated the strong impact of IFNγ produced from NK cells on IL-12-induced anti-tumor effects compared with that from non-NK cells.

Anti-tumor effects of IL-12 deteriorated slightly in mice depleted of NK cells

To examine the involvement of NK cells in the tumor deletion by IL-12 therapy, we induced depletion of NK cells by repeatedly injecting anti-asialoGM1 antibody. The cytolytic ability of NK cells was completely abolished in the anti-asialoGM1 antibody-injected group (Fig. 6a). Serum IFNγ induction by IL-12 in the NK depletion group was about half of that in the control immunoglobulin injected group (Fig. 6b). Unexpectedly, pCMV-IL-12 injection inhibited macroscopic liver metastasis of CT-26 cells in NK cell-depleted mice (Fig. 6c). However, a number of microscopic tumor regions were observed after IL-12 therapy in NK cell-depleted mice but not in control IgG-injected mice (Fig. 6d). This finding indicated that NK cells are required for a full-blown IL-12 anti-tumor effect, but IL-12′s anti-tumor effect was still observed even if the NK cells were knocked down. To examine the underlying mechanisms of anti-tumor effect in NK cell-depleted mice,

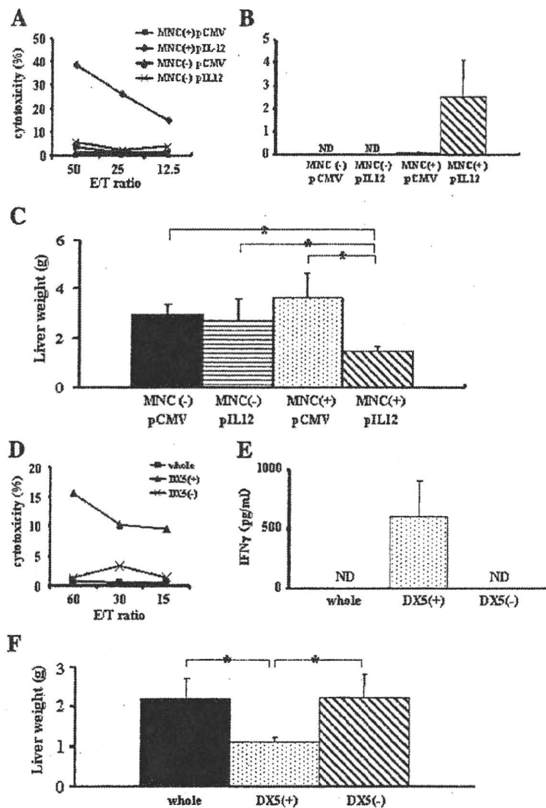

Fig. 5 Adoptive transfer of wild-type cells into GKO mice. Adoptive transfer of wild-type splenocytes restored anti-tumor effects of IL-12 in GKO mice. **a** GKO mice were intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. Yac1 lytic ability of hepatic mononuclear cells was expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **b** and **c** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice. Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **b** The levels of serum IFNγ 4 days after plasmid injection are expressed as mean and SD ($n = 6$/group). **c** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are indicated as mean and SD ($n = 6$/group). *ND* not detectable. $*p < 0.01$. Adoptive transfer of wild-type NK cells, but not non-NK cells, restored anti-tumor effects of IL-12 in GKO mice. **d** Wild-type splenocytes were purified into $DX5^+$ cells and $DX5^-$ cells. GKO mice were intravenously injected with 4.0×10^6 whole mononuclear cells or $DX5^+$ cells or $DX5^-$ cells and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after hydrodynamic injection. Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **e** and **f** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with whole mononuclear cells, $DX5^+$ cells or $DX5^-$ cells (4.0×10^6/mouse). Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **e** The levels of serum IFNγ are expressed as mean and SD ($n = 6$/group). **f** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are expressed as mean and SD ($n = 6$/group). *ND* not detectable. $*p < 0.001$

serum levels of IP-10 and MIG, chemokines downstream of IFNγ, were measured after IL-12 therapy (Fig. 6e). pCMV-IL-12-injected mice showed significant increase in both levels compared with pCMV-injected mice. Significant increase after pCMV-IL-12 injection was also found in NK cell-depleted mice, but not in GKO mice. This result suggests that production of these chemokines was not completely suppressed in NK cell-depleted mice in our experimental condition. Immunohistochemical analysis revealed that tumoral accumulation of CD4-positive cells and CD8-positive cells was observed in pCMV-IL-12-injected mice but not in pCMV-injected mice. On the other hand, similar levels of CD31 expression were observed in tumors of pCMV-injected mice and pCMV-IL-12-injected mice (Fig. 6d). These results suggest that IL-12's anti-tumor effects might be mediated by T-cell accumulating in the tumor rather than anti-angiogenesis.

Discussion

IL-12 is recognized as a master regulator of adaptive type 1, cell-mediated immunity. One major action of IL-12 is its induction of other cytokines, particularly IFNγ. A large amount of evidence has indicated that IL-12 administration leads to IFNγ production from a variety of immune cells, such as T cells [16], B cells [17], NK cells [18] and NKT cells [22]. The relative impact of each immune cell as the source of IFNγ has been controversial. The present study highlighted NK cells as a most efficient producer of IFNγ that is critical for IL-12-induced anti-tumor effects.

Flow cytometric analysis revealed higher in vivo production of IFNγ of NK cells than that of other cell types. The levels of serum IFNγ were around fourfold higher in Rag2 KO mice which only possess NK cells than in wild-type mice. On the other hand, NK-cell depletion in wild-type mice led to twofold reduction of serum IFNγ levels. These data indicate substantial contribution of NK cells in IFNγ production in vivo. Previous research has demonstrated that the specific cellular effects of IL-12 are due mainly to activation of STAT4 [23, 24]. IL-12-induced STAT4 phosphorylation leads to the production of IFNγ [25]. In agreement with these reports, our in vitro analysis showed that, in contrast to STAT1, STAT4 was directly phosphorylated upon IL-12 stimulation, being independent of IFNγ. Of interest is the finding that NK cells express higher levels of STAT4 than non-NK cells, suggesting that NK cells possess an ideal expression profile of STATs for producing IFNγ upon IL-12 stimulation. Indeed, in vitro analysis revealed that NK cells, upon IL-12 exposure, displayed higher levels of IFNγ production as well as STAT4 phosphorylation than non-NK cells. These in vitro

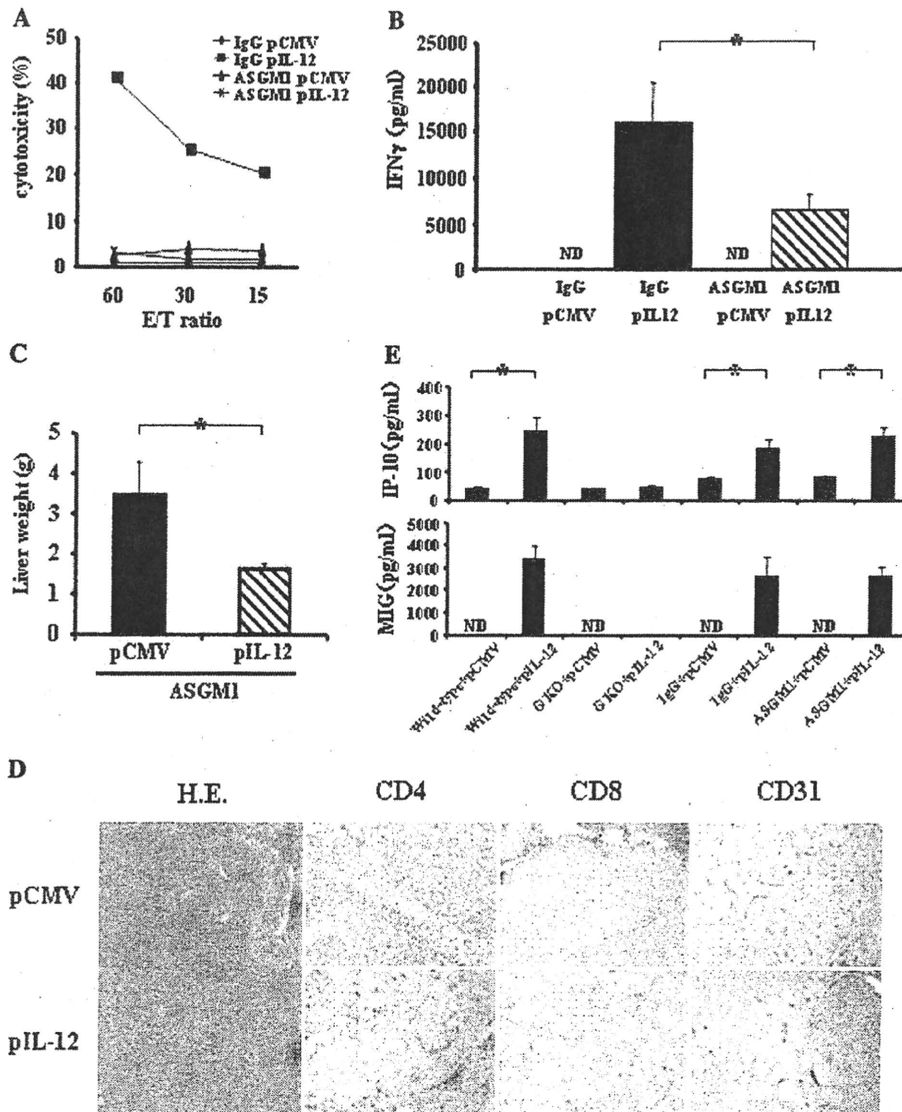

Fig. 6 Anti-tumor effects of IL-12 in NK-cell-depleted mice. Serum IFNγ levels and NK-cell activation. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and, 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. **a** Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFNγ are expressed as mean and SD ($n = 6$/group). *ND* not detectable. $*p < 0.005$. Anti-metastatic effects. Wild-type mice were intrasplenically injected with CT-26 cells and, 1 day later and then every 5 days, intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and hydrodynamically injected with either pCMV-IL-12 or pCMV 2 days after CT-26 injection. Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. **c** The results are indicated as mean and SD ($n = 6$/group). $*p < 0.001$. **d** Representative histology of liver sections analyzed by hematoxylin-eosin staining and immunohistochemistry of CD4, CD8 and CD31. **e** Serum levels of IP-10 and MIG. Wild-type or GKO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Four days later, each mice were bled to measure the levels of serum IP-10 and MIG. Results are expressed as mean and SD ($n = 6$/group). *ND* not detectable. $*p < 0.001$

data are consistent with the in vivo observation that NK cells are efficient producers of IFNγ during IL-12 therapy.

Many studies have demonstrated that IFNγ production is required for the anti-tumor effects of IL-12 [14, 26, 27]. In fact, we have demonstrated that deletion of IFNγ abolished NK cytotoxicity and the anti-metastatic effect of IL-12 therapy in the liver. A large amount of evidence supports the concept that a major action of IL-12 is to promote the differentiation of naïve CD4 + T cells into Th1 cells, which produce IFNγ. Previous research reported that CD4

T-cell depletion caused inhibition of anti-tumor effects. More recent studies have supported a critical role of IFNγ as a third signal for CD8 T-cell differentiation. There have been many reports focusing on IFNγ production from T cells induced by IL-12 for the anti-tumor effect of IL-12 [28]. Segal et al. performed an elegant study showing a critical role of T-cell production of IFNγ in the anti-tumor effect by adoptively transferring T cells into GKO mice in a subcutaneous tumor model [29]. However, apart from this study, little is known about the contribution of each immune cell as a producer of IFNγ in terms of an anti-tumor effect. In our model, T-cell mediated adaptive responses were not required for the anti-metastatic effect of IL-12. More importantly, the anti-metastatic effects of IL-12 were restored in GKO mice by an adoptive transfer of wild-type NK cells. The same number of non-NK cells could not provoke IL-12-induced anti-tumor effects in GKO mice. The present study demonstrated for the first time a potent effect of NK cells on producing IFNγ that was critical for anti-metastatic effect during IL-12 therapy.

Our study showed that the main IFNγ producer of IL-12 was NK cells. So we focused on NK cells which were activated by IL-12 in an IFNγ-dependent manner to examine the cellular mechanism of protection against hepatic metastasis. Many studies have shown the importance of each subset (NK- [12], NKT- [10] and T [9, 30] cells) for anti-tumor effects of IL-12. In the present study, NK cells were sufficient while T cells, B cells, NKT cells were dispensable for IL-12-mediated NK-cell activation and anti-metastatic effects as IL-12 therapy showed Yac1 lytic ability and antimetastatic effects in Rag2 KO mice. On the other hand, NK-cell depletion by a repeated injection of anti-aialoGM1 antibody protected wild-type mice from macroscopic liver metastasis, but did not from microscopic liver metastasis. Thus, although NK cells were required for a full-blown IL-12 anti-tumor effect, other anti-tumor pathways are activated by IL-12 in the absence of NK cells. Serum levels of IP-10 and MIG suggest that production of these chemokines downstream of IFNγ was not suppressed in NK-cell-depleted mice in our experimental condition. When compared with the experiment on GKO mice, accumulation of CD4-positive cells and CD8-positive cells were more evident in NK-cell-depleted mice than in GKO mice (Supplementary Figure). On the other hand, there was no remarkable difference in the expression of CD31 between pCMV injection and pCMV-IL-12 injection. These results suggested that in NK-cell-depleted mice IL-12 may exert anti-tumor effect via T-cell accumulation rather than anti-angiogenesis.

Since the liver contains an abundance of immune cells (especially NK cells) [31], the cytokine-mediated activation of these cells may be a promising approach toward anti-tumor therapy in this organ [32]. IL-12 is a cytokine known to elicit a potent anti-tumor effect in mouse experimental models. However, clinical trials attempted to date were interrupted by fatal adverse effects. Systemic IL-12 therapy has been associated with dose-limiting toxicity [33]. IL-12 induces activation of the pro-inflammatory pathway which causes the complications of high dose cytokine, independent of the action of IFNγ [34]. On the other hand, the levels of immunosuppressive cytokine, for example, TGF-β1 or IL-10 were significantly higher in patients with hepatocellular cancer and colon cancer [35–38]. In particular, TGF-β1 in serum can limit NK-cell IFNγ production [39]. Thus, in patients with advanced disease, IL-12 may not be able to exert its potent anti-tumor immune-effects because IFNγ, which is an important mediator of the IL-12-induced immune response, is less effective in a tumor environment. In the present study, we demonstrated that NK-cell IFNγ production induced by IL-12 was sufficient for the anti-metastatic effect of IL-12 in the liver. Thus, a strategy of efficiently producing IFNγ from NK cells may be important for avoiding toxicity of IL-12 therapy.

IL-12 gene therapy has an advantage to allow local production of the cytokine at the tumor sites with low serum concentration. Studies demonstrated that intratumoral administration of adenovirus encoding IL-12 to animals with different types of carcinoma caused complete tumor eradication and increased long-term survival [40, 41]. Moreover, injection of IL-12-encoding adenovirus in one nodule of liver tumor resulted in regression of distant nodules in the liver [41]. However, in a clinical trial anti-tumor activity of IL-12-encoding adenovirus was only observed in the injected tumor sites, but not in distant tumors [42]. The present study shed light on hydrodynamic transfection of hepatocytes as a promising strategy to eradicate disseminated tumors from whole liver.

In summary, NK cells are not just an effector for innate immunity but a mediator producing IFNγ that is critical for the IL-12 anti-tumor effects. Extremely higher expression of STAT4 may be a basis for efficient production of IFNγ from NK cells.

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References

1. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G (1989) Identification and purification of natural killer cell stimulatory factor

(NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med 170(3):827–845
2. Stern AS, Podlaski FJ, Hulmes JD, Pan YC, Quinn PM, Wolitzky AG, Familletti PC, Stremlo DL, Truitt T, Chizzonite R, Gately MK (1990) Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc Natl Acad Sci USA 87(17):6808–6812
3. Watford WT, Moriguchi M, Morinobu A, O'Shea JJ (2003) The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev 14(5):361–368
4. Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3(2):133–146
5. Colombo MP, Trinchieri G (2002) Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev 13(2):155–168
6. Del Vecchio M, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G, Anichini A (2007) Interleukin-12: biological properties and clinical application. Clin Cancer Res 13(16):4677–4685
7. Wigginton JM, Gruys E, Geiselhart L, Subleski J, Komschlies KL, Park JW, Wiltrout TA, Nagashima K, Back TC, Wiltrout RH (2001) IFN-gamma and Fas/FasL are required for the antitumor and antiangiogenic effects of IL-12/pulse IL-2 therapy. J Clin Invest 108(1):51–62
8. Lee JC, Kim DC, Gee MS, Saunders HM, Sehgal CM, Feldman MD, Ross SR, Lee WM (2002) Interleukin-12 inhibits angiogenesis and growth of transplanted but not in situ mouse mammary tumor virus-induced mammary carcinomas. Cancer Res 62(3):747–755
9. Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, Wolf SF, Gately MK (1993) Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J Exp Med 178(4):1223–1230
10. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M (1997) Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science 278(5343):1623–1626
11. Zilocchi C, Stoppacciaro A, Chiodoni C, Parenza M, Terrazzini N, Colombo MP (1998) Interferon gamma-independent rejection of interleukin 12-transduced carcinoma cells requires CD4 + T cells and Granulocyte/Macrophage colony-stimulating factor. J Exp Med 188(1):133–143
12. Kodama T, Takeda K, Shimozato O, Hayakawa Y, Atsuta M, Kobayashi K, Ito M, Yagita H, Okumura K (1999) Perforin-dependent NK cell cytotoxicity is sufficient for anti-metastatic effect of IL-12. Eur J Immunol 29(4):1390–1396
13. Takeda K, Hayakawa Y, Atsuta M, Hong S, Van Kaer L, Kobayashi K, Ito M, Yagita H, Okumura K (2000) Relative contribution of NK and NKT cells to the anti-metastatic activities of IL-12. Int Immunol 12(6):909–914
14. Ogawa M, Yu WG, Umehara K, Iwasaki M, Wijesuriya R, Tsujimura T, Kubo T, Fujiwara H, Hamaoka T (1998) Multiple roles of interferon-gamma in the mediation of interleukin 12-induced tumor regression. Cancer Res 58(11):2426–2432
15. Subleski JJ, Hall VL, Back TC, Ortaldo JR, Wiltrout RH (2006) Enhanced antitumor response by divergent modulation of natural killer and natural killer T cells in the liver. Cancer Res 66(22):11005–11012
16. Kubin M, Kamoun M, Trinchieri G (1994) Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. J Exp Med 180(1):211–222
17. Yoshimoto T, Okamura H, Tagawa YI, Iwakura Y, Nakanishi K (1997) Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-gamma production from activated B cells. Proc Natl Acad Sci USA 94(8):3948–3953
18. Lauwerys BR, Renauld JC, Houssiau FA (1999) Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. Cytokine 11(11):822–830
19. Takehara T, Uemura A, Tatsumi T, Suzuki T, Kimura R, Shiotani A, Ohkawa K, Kanto T, Hiramatsu N, Hayashi N (2007) Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNalpha gene to mice. Int J Cancer 120(6):1252–1260
20. Watanabe M, Fenton RG, Wigginton JM, McCormick KL, Volker KM, Fogler WE, Roessler PG, Wiltrout RH (1999) Intradermal delivery of IL-12 naked DNA induces systemic NK cell activation and Th1 response in vivo that is independent of endogenous IL-12 production. J Immunol 163(4):1943–1950
21. Takehara T, Suzuki T, Ohkawa K, Hosui A, Jinushi M, Miyagi T, Tatsumi T, Kanazawa Y, Hayashi N (2006) Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication. J Hepatol 44(2):267–274
22. Shin T, Nakayama T, Akutsu Y, Motohashi S, Shibata Y, Harada M, Kamada N, Shimizu C, Shimizu E, Saito T, Ochiai T, Taniguchi M (2001) Inhibition of tumor metastasis by adoptive transfer of IL-12-activated Valpha14 NKT cells. Int J Cancer 91(4):523–528
23. Thierfelder WE, van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DA, Doherty PC, Grosveld GC, Ihle JN (1996) Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature 382(6587):171–174
24. Kaplan MH, Sun YL, Hoey T, Grusby MJ (1996) Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. Nature 382(6587):174–177
25. Morinobu A, Gadina M, Strober W, Visconti R, Fornace A, Montagna C, Feldman GM, Nishikomori R, O'Shea JJ (2002) STAT4 serine phosphorylation is critical for IL-12-induced IFN-gamma production but not for cell proliferation. Proc Natl Acad Sci USA 99(19):12281–12286
26. Comes A, Di Carlo E, Musiani P, Rosso O, Meazza R, Chiodoni C, Colombo MP, Ferrini S (2002) IFN-gamma-independent synergistic effects of IL-12 and IL-15 induce anti-tumor immune responses in syngeneic mice. Eur J Immunol 32(7):1914–1923
27. Hafner M, Falk W, Echtenacher B, Mannel DN (1999) Interleukin-12 activates NK cells for IFN-gamma-dependent and NKT cells for IFN-gamma-independent antimetastatic activity. Eur Cytokine Netw 10(4):541–548
28. Komita H, Homma S, Saotome H, Zeniya M, Ohno T, Toda G (2006) Interferon-gamma produced by interleukin-12-activated tumor infiltrating CD8 + T cells directly induces apoptosis of mouse hepatocellular carcinoma. J Hepatol 45(5):662–672
29. Segal JG, Lee NC, Tsung YL, Norton JA, Tsung K (2002) The role of IFN-gamma in rejection of established tumors by IL-12: source of production and target. Cancer Res 62(16):4696–4703
30. Nastala CL, Edington HD, McKinney TG, Tahara H, Nalesnik MA, Brunda MJ, Gately MK, Wolf SF, Schreiber RD, Storkus WJ, Lotze MT (1994) Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. J Immunol 153(4):1697–1706
31. Doherty DG, O'Farrelly C (2000) Innate and adaptive lymphoid cells in the human liver. Immunol Rev 174:5–20
32. Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, Hiraide H (2000) The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag + T cells in T helper 1 immune responses. Immunol Rev 174:35–46
33. Car BD, Eng VM, Lipman JM, Anderson TD (1999) The toxicology of interleukin-12: a review. Toxicol Pathol 27(1):58–63
34. Biber JL, Jabbour S, Parihar R, Dierksheide J, Hu Y, Baumann H, Bouchard P, Caligiuri MA, Carson W (2002) Administration of

two macrophage-derived interferon-gamma-inducing factors (IL-12 and IL-15) induces a lethal systemic inflammatory response in mice that is dependent on natural killer cells but does not require interferon-gamma. Cell Immunol 216(1–2):31–42
35. Tsushima H, Ito N, Tamura S, Matsuda Y, Inada M, Yabuuchi I, Imai Y, Nagashima R, Misawa H, Takeda H, Matsuzawa Y, Kawata S (2001) Circulating transforming growth factor beta 1 as a predictor of liver metastasis after resection in colorectal cancer. Clin Cancer Res 7(5):1258–1262
36. Okumoto K, Hattori E, Tamura K, Kiso S, Watanabe H, Saito K, Saito T, Togashi H, Kawata S (2004) Possible contribution of circulating transforming growth factor-beta1 to immunity and prognosis in unresectable hepatocellular carcinoma. Liver Int 24(1):21–28
37. Chau GY, Wu CW, Lui WY, Chang TJ, Kao HL, Wu LH, King KL, Loong CC, Hsia CY, Chi CW (2000) Serum interleukin-10 but not interleukin-6 is related to clinical outcome in patients with resectable hepatocellular carcinoma. Ann Surg 231(4):552–558
38. Galizia G, Lieto E, De Vita F, Romano C, Orditura M, Castellano P, Imperatore V, Infusino S, Catalano G, Pignatelli C (2002) Circulating levels of interleukin-10 and interleukin-6 in gastric and colon cancer patients before and after surgery: relationship with radicality and outcome. J Interferon Cytokine Res 22(4):473–482
39. Meadows SK, Eriksson M, Barber A, Sentman CL (2006) Human NK cell IFN-gamma production is regulated by endogenous TGF-beta. Int Immunopharmacol 6(6):1020–1028
40. Caruso M, Pham-Nguyen K, Kwong YL, Xu B, Kosai KI, Finegold M, Woo SL, Chen SH (1996) Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. Proc Natl Acad Sci USA 93(21):11302–11306
41. Barajas M, Mazzolini G, Genove G, Bilbao R, Narvaiza I, Schmitz V, Sangro B, Melero I, Qian C, Prieto J (2001) Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. Hepatology 33(1):52–61
42. Sangro B, Mazzolini G, Ruiz J, Herraiz M, Quiroga J, Herrero I, Benito A, Larrache J, Pueyo J, Subtil JC, Olague C, Sola J et al (2004) Phase I trial of intratumoral injection of an adenovirus encoding interleukin-12 for advanced digestive tumors. J Clin Oncol 22(8):1389–1397

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Factors affecting efficacy in patients with genotype 2 chronic hepatitis C treated by pegylated interferon alpha-2b and ribavirin: reducing drug doses has no impact on rapid and sustained virological responses

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SUMMARY. Reducing the dose of drug affects treatment efficacy in pegylated interferon (Peg-IFN) and ribavirin combination therapy for patients with hepatitis C virus (HCV) genotype 1. The aim of this study was to investigate the impact of drug exposure, as well as the baseline factors and the virological response on the treatment efficacy for genotype 2 patients. Two-hundred and fifty patients with genotype 2 HCV who were to undergo combination therapy for 24 weeks were included in the study, and 213 completed the treatment. Significantly more patients who achieved a rapid virological response (RVR), defined as HCV RNA negativity at week 4, achieved a sustained virological response (SVR) (92%, 122/133) compared with patients who failed to achieve RVR (48%, 38/80) ($P < 0.0001$). Multivariate logistic-regression analysis showed that only platelet counts [odds ratio (OR), 1.68; confidence interval (CI), 1.002–1.139] and RVR (OR, 11.251; CI, 5.184–24.419) were independently associated with SVR, with no correlation being found for the mean dose of Peg-IFN and ribavirin for RVR and SVR. Furthermore, in the stratification analysis of the timing of viral clearance, neither mean dose of Peg-IFN ($P = 0.795$) nor ribavirin ($P = 0.649$) affected SVR in each group. Among the patients with RVR, the lowest dose group of Peg-IFN (0.77 ± 0.10 μg/kg/week) and ribavirin (6.9 ± 0.90 mg/kg/day) showed 100% and 94% of SVR. Hence, RVR served as an important treatment predictor, and drug exposure had no impact on both SVR and RVR in combination therapy for genotype 2 patients.

Keywords: chronic hepatitis C, drug exposure, genotype 2, peginterferon and ribavirin combination therapy.

INTRODUCTION

The current standard of care for chronic hepatitis C (CHC) patients consists of combination therapy using pegylated interferon (Peg-IFN) and ribavirin [1–3]. Large, randomized clinical trials have demonstrated that 42–52% of hepatitis C virus (HCV) genotype 1 'difficult-to-treat' patients achieved sustained virological response (SVR), whereas 76–84% of HCV genotype 2 or 3 infected patients treated with Peg-IFN and ribavirin achieved SVR [4–6]. It also has been shown that in HCV genotype 2 and 3 infected patients, 24-week treatment regimens are just as effective as 48-week regimens [6,7]. Therefore, current guidelines recommend a 24-week treatment for these patients in contrast to 48 weeks for genotype 1 patients [1–3]. However, as side effects are common and treatment is expensive for this therapy, it would be ideal to be able to further reduce the total amount of drug medication

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CHC, chronic hepatitis C; c-EVR, complete early virological response; ETR, end of treatment response; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; IFN, interferon; NPV, negative predictive value; Peg-IFN, pegylated interferon; RVR, rapid virological response; SVR, sustained virological response.

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without loss of treatment efficacy for genotype 2 and 3 patients.

In HCV genotype 1 patients, reducing drug doses affects treatment efficacy. In our investigation of HCV genotype 1 patients, the rate of complete early virological response (c-EVR), defined as HCV RNA negativity at week 12, was affected by the mean dose of Peg-IFN during the first 12 weeks dose-dependently ($P < 0.0001$) [8]. Furthermore, we showed that only 4% relapse was found in patients given ≥12 mg/kg/day of ribavirin among those with c-EVR, and the relapse rate showed a decline in relation to the increase in the dose of ribavirin ($P = 0.0002$) [9]. On the contrary, it remains to be determined whether treatment efficacy can be preserved by further reducing both drug doses in genotype 2 and 3 patients. Because lower doses are expected to cause fewer adverse effects, it is important to find whether reduced drug doses can be used while retaining efficacy.

In the present study, we retrospectively evaluated the efficacy of Peg-IFN alpha-2b and ribavirin combination therapy for 24 weeks in patients infected with HCV genotype 2 and analysed the factors that affected the treatment efficacy, with particular interests in the drug impact of Peg-IFN and ribavirin.

PATIENTS AND METHODS

Patient selection and study design

Patients considered to be eligible for this study were those infected with HCV genotype 2 who underwent Peg-IFN alpha-2b (Schering-Plough K.K., Tokyo, Japan) and ribavirin (Schering-Plough K.K.) combination therapy from December 2005 to July 2007 at 29 medical institutions taking part in the Osaka Liver Forum and had completed the 24-week observation after a clinical course of 24 weeks. Patients with the following criteria were excluded: hepatitis B virus or human immunodeficiency virus coinfection, decompensated liver disease, severe cardiac, renal, haematological or chronic pulmonary disease, poorly controlled psychiatric disease, poorly controlled diabetes and immunologically mediated disease. Liver biopsy had been performed within 24 months prior to the treatment, and histological results were classified according to the METAVIR scoring system [10].

Written informed consent was obtained from each patient, and the study protocol was reviewed and approved according to the ethical guidelines of the 1975 Declaration of Helsinki by institutional review boards at the respective sites.

Patients were treated with Peg-IFN alpha-2b plus ribavirin for the duration of the study of 24 weeks. Peg-IFN alpha-2b and ribavirin dosages were based on body weight according to the manufacturer's instructions: Peg-IFN alpha-2b was given subcutaneously weekly (45 kg or less, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; 91 kg or more, 150 μg/dose), and ribavirin was given orally daily (60 kg or less, 600 mg/day; 61–80 kg, 800 mg/day; 81 kg or more, 1000 mg/day). The drug doses were also modified based on the manufacturer's instructions according to the intensity of the haematologic adverse effects.

Virological tests

Serum HCV RNA level was quantified by PCR assay (COBAS Amplicor HCV Test v2.0, Chugai-Roche Diagnostics, Tokyo, Japan), with a sensitivity limit of 5000 IU/mL and a dynamic range from 5000 to 5 000 000 IU/mL [11].

Serum HCV RNA was assessed by qualitative PCR assay (COBAS Amplicor HCV Monitor Test v2.0, Chugai-Roche Diagnostics), with a detection limit of 50 IU/mL [12].

Assessment of efficacy

Serum HCV RNA (qualitatively or quantitatively) was measured at weeks 4, 8, 12 and 24 during treatment and after 24 weeks of follow-up without treatment. Patients were classified as having a rapid virological response (RVR) if serum HCV RNA was undetectable (<50 IU/mL) at week 4 and at the end of treatment response (ETR) at week 24 of treatment. SVR was defined as undetectable HCV RNA at week 24 after treatment. Patients with an ETR who sero-reverted to HCV RNA during follow-up were classified as relapsers.

Drug exposure

The amounts of Peg-IFN alpha-2b and ribavirin actually taken by each patient during the treatment period were evaluated by reviewing the medical records. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline; Peg-IFN alpha-2b expressed as μg/kg/week and ribavirin as mg/kg/day.

Data collection

The medical records were retrospectively reviewed and the factors necessary for this examination were extracted: age, sex, body weight, body mass index (BMI), basic laboratory assessments, liver histology, quantitative and qualitative HCV RNA, dose of Peg-IFN alpha-2b and ribavirin received at each administration, and the response to treatment.

Statistical analysis

This study was a retrospective study and, for treatment results and the analysis of related factors, analysis was carried out only for cases in which the treatment had been completed (per-protocol analysis). Continuous variables are reported as the mean with standard deviation (SD) or

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median level, while categorical variables are shown as the count and proportion. In univariate analysis, the Mann–Whitney *U*-test was used to analyse continuous variables, while chi-squared and Fisher's exact tests were used for analysis of categorical data. Variables with $P < 0.05$ at univariate analysis were retained for the multivariate logistic-regression analysis. Stepwise and multivariate logistic-regression models were used to explore the independent factors that could be used to predict a virological response. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. For all tests, two-sided *P*-values were calculated and the results were considered statistically significant if $P < 0.05$. Statistical analysis was performed using the SPSS program for Windows, version 15.0J (SPSS, Chicago, IL, USA).

RESULTS

The baseline characteristics for the total cohort are shown in Table 1. Most of the patients were female (56%) with a mean age of 54 years. Seventy per cent of the patients were treatment naïve. Of the 250 patients, liver biopsies were performed for 174 patients, and 18 of them had advanced fibrosis (F 3–4).

Of the total of 250 patients, 37 (15%) were withdrawn from treatment because of adverse events: decreased haemoglobin ($n = 10$), psychiatric problems including depression ($n = 9$), fatigue ($n = 3$), thrombocytopenia, neutropenia, pyrexia, rash, cerebral haemorrhage, bleeding of ocular fundus, dyspnea, dizziness, jaundice, transaminase rise, gastrointestinal symptoms ($n = 1$) and other adverse events ($n = 4$). Eight of these patients who discontinued treatment prematurely had SVR (8/37; 22%).

Drug adherence

Seventy-nine of the 213 patients (37%) required dose reduction of Peg-IFN alpha-2b, 99 (46%) of ribavirin because of adverse events (not including patients who later discontinued treatment because of adverse event). Neutropenia (24/79; 30%) and thrombocytopenia (24/79; 30%) were the most common adverse events for dose reduction of Peg-IFN alpha-2b, and decreased haemoglobin (82/99; 83%) for that of ribavirin.

Virological response

Of the 213 patients who completed 24 weeks of treatment and 24 weeks of follow-up, 160 (75%) patients were clear of HCV RNA at week 4, 191 (90%) at week 8, 196 (92%) at week 12. ETR was observed for 195 (92%), and SVR for 160 (75%). The relapse rate was 18% (35/195).

Virological response according to the timing of viral clearance

Positive and negative prediction of sustained virological response according to the timing of viral clearance

We examined SVR rates according to the timing of viral clearance for the case in which HCV RNA was cleared during the treatment (Fig. 1a). The SVR rate was 92% (122/133) for patients clear of HCV RNA until week 4, 64% (37/58) from week 5 until week 8, 20% (1/5) from week 9 until

Table 1 Baseline demographic and viral characteristics of patients

Number of cases	250	
Age (years)*	54.0 ± 12.4	(22–76)
Sex (male/female)	110/140	
Body weight (kg)*	60.3 ± 11.7	(39–99)
Body mass index (kg/m^2)*	23.1 ± 3.2	(16–35)
Past IFN therapy (naïve/experienced)†	175/70	
HCV RNA (KIU/mL)‡	1700	(4–5000 <)
Fibrosis (0/1/2/3/4)§	18/98/40/14/4	
Activity (0/1/2/3)§	15/81/70/8	
White blood cells (/mm^3)*	5210 ± 1,750	(2100–13 870)
Neutrophils (/mm^3)*	2700 ± 1,250	(590–9020)
Red blood cells (×10^4/mm^3)*	436 ± 48	(307–554)
Haemoglobin (g/dL)*	13.9 ± 1.4	(10–18)
Platelets (×10^4/mm^3)*	18.3 ± 6.4	(4–41)
ALT (IU/L)*	79 ± 77	(13–581)
γ-GTP (U/L)*	56 ± 65	(7–479)
Creatinine(mg/dL)*	0.7 ± 0.1	(0.4–1.1)

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase. *Values expressed as mean ± SD (range), †interferon treatment history was not known for five patients, ‡values expressed as median (range), §data for 76 patients are missing.

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