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REVIEW

The role of microRNAs in hepatocarcinogenesis: current knowledge and future prospects

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Abstract MicroRNAs (miRNAs) are small, noncoding RNA molecules that regulate gene expression post-transcriptionally through complementary base pairing with thousands of messenger RNAs. Although the precise biological functions of individual miRNAs are still unknown, miRNAs are speculated to play important roles in diverse biological processes through fine regulation of their target gene expression. A growing body of data indicates the deregulation of miRNAs during hepatocarcinogenesis. In this review, we summarize recent findings regarding deregulated miRNA expression and their possible target genes in hepatocarcinogenesis, with emphasis on inflammation-related hepatocarcinogenesis. Because miRNA-based strategies are being applied to clinical therapeutics, precise knowledge of miRNA functions is crucial both scientifically and clinically. We discuss the current open questions from these points of view, which must be clarified in the near future.

Keywords MicroRNA · Hepatocarcinogenesis · Inflammation

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Introduction

MicroRNAs (miRNAs) are short, single-stranded, noncoding RNAs, which are expressed in most organisms, from plants to vertebrates [1]. Since the discovery of the miRNA lin-4 in *Caenorhabditis elegans* [2, 3], 1,872 miRNA precursors and 2,578 mature miRNA sequences in humans have been deposited in miRBase, a public repository hosted by the Sanger Institute, as of November 2013 [4]. Bioinformatic predictions suggest that miRNAs regulate more than 30 % of human protein-coding genes [5–7]. Through the regulation of gene expression, miRNAs are involved in various physiological and pathological processes, including cell proliferation, apoptosis, differentiation, metabolism, oncogenesis and oncogenic suppression [8, 9]. Thus, it is not surprising that deregulation of miRNAs is linked closely to various human pathological conditions. In this review, we will describe the crucial role of miRNAs in liver carcinogenesis, especially inflammation-related hepatocarcinogenesis.

Biogenesis and functions of miRNAs

Transcription is the first step in miRNA expression (Fig. 1). Similar to most protein-coding genes, transcriptional factors, enhancers and silencers are involved in miRNA transcription [10–12]. Epigenetic mechanisms, such as promoter methylation or histone modification, also regulate miRNA transcription, and it was shown that histone deacetylase (HDAC) inhibition results in transcriptional changes in ~40 % of miRNAs [13].

Primary miRNAs, which possess stem-loop structures, are transcribed by RNA polymerase II [8]. These pri-miRNAs are processed by a microprocessor complex

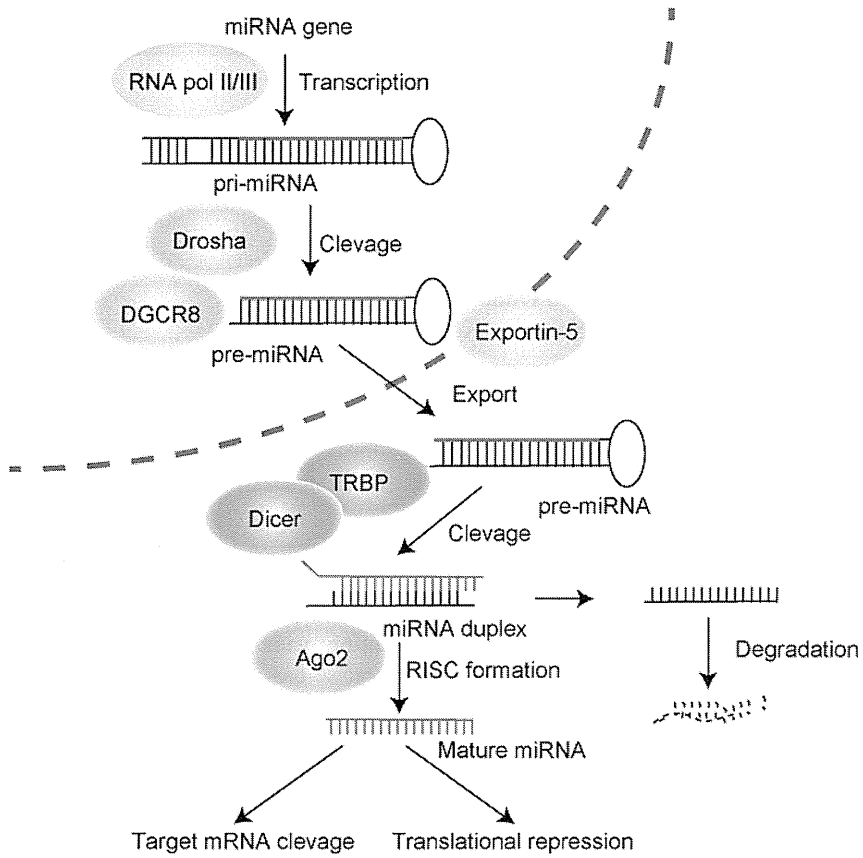

Fig. 1 Biogenesis of miRNAs. The primary miRNA transcript (pri-miRNA) is transcribed from the genome by RNA polymerase II or III. The microprocessor complex Drosha–DGCR8 cleaves the pri-miRNA into the precursor hairpin, pre-miRNA in the nucleus. The pre-miRNA is exported from the nucleus by exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein, TRBP, cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage or translational repression. The passenger strand (*black*) is degraded

comprising Drosha (RNAase III) [14] and DGCR8/Pasha [15] in the nucleus [16]. The processed products are approximately 65-nucleotide hairpin-shaped precursors (pre-miRNAs) that are transported to the cytoplasm via exportin-5 [17, 18]. Pre-miRNAs are further cleaved into mature miRNAs by Drosha and Dicer RNA polymerase III. Mature miRNA duplexes are loaded onto an RNA-induced silencing complex (RISC) and are unwound into the single-stranded mature form [19–21]. The resulting co-complex directly targets the 3′-untranslated regions (3′-UTRs) of target mRNAs, depending on the sequence similarities, to negatively regulate their expression by enhancing mRNA cleavage or inhibiting translation (Fig. 1) [8, 22]. Because most miRNAs guide the recognition of imperfect matches of target mRNAs, individual miRNAs have multiple (probably hundreds) of mRNA targets. In addition, multiple miRNAs can cooperate to regulate the expression of the same transcript [6]. Thus, depending upon the identity of the target mRNAs, miRNAs play roles as "fine-tuners of gene expression" in the control of various biological functions.

Identifying functionally important miRNA target genes is crucial for understanding the impact of specific miRNAs on cellular function. However, this is challenging because miRNAs usually have imperfect complementarity with their targets [22]. In mammals, the most consistent requirement for miRNA-target interaction, although not always essential, is a contiguous and perfect pairing of the miRNA (nt 2–8), representing the "seed" sequence [22]. In many cases, the seed sequences determine this recognition, but in other cases, additional determinants are required, such as reasonable complementarity to the miRNA 3′ half to stabilize the interaction. In addition, target pairing to the center of some miRNAs has also been reported [23]. Although public miRNA target prediction algorithms, such as TargetScan [24] and PicTar [25], have facilitated the rapid identification of miRNA target genes [22], candidates should be validated experimentally.

miRNAs and cancer

The involvement of miRNAs in cancer pathogenesis is well established. miRNAs can affect six hallmarks of malignant cells, which are (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) angiogenesis, and (6) invasion and metastasis [26]. miRNAs are frequently

up- or downregulated in malignant tissues and can be considered oncogenes or tumor suppressors, respectively. However, it is essential to test experimentally whether the deregulated miRNAs are actually causative to carcinogenesis, since miRNAs have a very restricted tissue-specific expression and the apparent miRNA modulation in cancer tissues may only reflect the different constituents of a cell population as compared to normal tissues. Extensive analyses have confirmed the causative roles of miRNAs in cancer by using either human cancer cells or genetically engineered animal models, such as transgenic expression of miR-155, miR-21 and miR-15-a/16-1, which are sufficient to initiate lymphomagenesis in mice [27–29]. These results suggest the potential role of miRNAs in the pathogenesis of carcinogenesis and as therapeutic targets.

miRNAs and hepatocarcinogenesis

Numerous reports regarding the deregulated expression of miRNAs in human hepatocellular carcinoma (HCC) are extant. Most studies compared the miRNA expression levels between cancer tissues and background non-tumorous tissues, selected candidate miRNA(s) and revealed their target genes, which may be involved in carcinogenesis. As shown in Tables 1 and 2, many miRNAs have been identified as downregulated or upregulated in recent studies (Tables 1, 2). However, these numerous results are not always superimposable due to the large variances in the results. These significant differences may be due to several reasons, such as the use of different techniques or different samples as controls, normal liver tissues versus peritumoral non-neoplastic tissues. In addition, one may need to take into consideration the fact that HCCs arise in background livers with different etiologies, such as hepatitis B, hepatitis C or steatohepatitis, and also the age or sex of the tissue-derived patients and background liver condition, such as fibrosis staging or inflammation activity, which may result in differences in the expression status of miRNAs. Despite these considerable limitations, the list suggests that diverse miRNAs play crucial roles in hepatocarcinogenesis. We will briefly describe some of them below.

The expression levels of miRNAs have restricted tissue specificities. In the liver, miR-122, miR-192 and miR-199a/b-3p are the three most expressed miRNAs, accounting for 52, 17 and 5 % of all mRNAs in the tissues, respectively [30]. The tumorigenic role of the loss of miR-122 was confirmed in gene-knockout mice [31, 32] and its expression is indeed decreased in half of the HCCs, especially non-viral HCCs [30]. We also reported that decreased expression of miR-122 is linked with poor prognosis of HCC [33]. While miR-192 does not appear to be deregulated in HCC samples in previous studies, miR-199a/b-3p is decreased with high frequency in HCC, which is closely linked to a poor prognosis of HCC [30]. In contrast, miR-21, whose expression is increased following rat hepatectomy [34], is upregulated as a known oncomiRNA and represses PTEN signaling, resulting in promotion of HCC development [35]. Although individual miRNAs may be involved in hepatocarcinogenesis, because miRNAs often function co-operatively, the extent of their involvement remains to be determined.

As described above, miRNAs usually have multiple mRNA targets. Thus, it is not practical to describe only a few genes as being responsible for the phenotypes by deregulation of specific miRNAs, while many studies identify specific genes as targets of specific miRNAs. Nonetheless, the identified targeted genes are generally related to at least one of the hallmarks of cancer, such as cell growth, apoptosis, invasion, and so on. These results suggest that the deregulation of miRNA expression might mediate hepatocarcinogenesis through deregulating the expression of their target genes.

The miRNAs identified as deregulated in hepatocarcinogenesis may be useful as diagnostic and prognostic markers [36], because miRNAs in the circulation are reported to be relatively stable [37]. Also, deregulated miRNAs may be candidate therapeutic and preventive targets against HCC. However, to include the obtained results in clinical interventional applications, it is necessary to confirm if the deregulated miRNAs are truly drivers or are simply passive in hepatocarcinogenesis. To this end, genetically modified mice may provide some information. In addition, to correctly interpret the data, a standard method of normalizing the microRNAome data between studies may also be crucial. Since there are multiple target genes of miRNAs and, conversely, one transcript can be targeted by multiple miRNAs, a more systematic comparison using miRNA data, transcriptome data and proteome data would increase our understanding of the consequences of the deregulation of miRNAs during hepatocarcinogenesis. From this point of view, systematic and comprehensive target gene analyses for in silico systems biology models may be one option to resolve these issues.

miRNAs linked to inflammation-mediated hepatocarcinogenesis

Inflammation is considered to be a major cause of cancer [38, 39]. In the liver, hepatocarcinogenesis frequently occurs in persistently inflamed liver tissues caused by chronic hepatitis viral infection or non-alcoholic steatohepatitis. However, the molecular linkage between chronic inflammation and carcinogenesis is not well characterized.

Table 1 Upregulated miRNAs in hepatocarcinogenesis

miRNA	Expression levels	Targets	Main tested samples	References
miR-17-5p	Upregulated	p38 pathway	Cultured cells, human tissues	[52]
miR-18a	Upregulated	ER1a	Human tissues, cultured cells	[53]
miR-21	Upregulated	C/EBPb	Mouse CDAA model	[54]
	Upregulated	PTEN	Human tissues, cultured cells	[35]
miR-22	Upregulated	ERa, IL-1a	Human tissues, cultured cells, DEN model	[55]
miR-23a	Upregulated	PGC-1a,G6PC	Human tissues, cultured cells	[56]
miR-26a	Upregulated	Lin28B, Zcchc11	Human tissues, xenograft model	[57]
	Upregulated	NF-κB, IL-6 pathways	Human tissues	[58]
miR-30d	Upregulated	GNAI2	Human tissues, cultured cells	[59]
miR-100	Upregulated		Human tissues	[60]
miR-106b	Upregulated	APC	Human tissues, cultured cells	[61]
miR-122	Upregulated		Human tissues	[60]
miR-130b	Upregulated	TP53INP1	Human tissues, xenograft model	[62]
miR-135a	Upregulated	FOXM1, MTSS1	Human tissues, cultured cells, xenograft	[63]
miR-143	Upregulated	FNDC3B	Human tissues, HBX transgenic mouse	[64]
miR-146a	Upregulated in endothelial cells	BRCA, PDGFRA	Cultured cells	[65]
miR-151	Upregulated	FAK	Human tissues, cultured cells	[66]
	Upregulated	FAK, RhoGDIA	Human tissues, cultured cells	[67]
miR-155	Upregulated	SOCS1	Orthotropic transplant model	[68]
	Upregulated	DKK1, APC	Human tissues, cultured cells	[69]
	Upregulated	PTEN	Mouse CDAA model	[54]
miR-181	Upregulated	TIMP3	Mouse CDAA model	[70]
	Upregulated	CDX2, GATA6, NLK	Cultured cells	[71]
miR-183	Upregulated	AKAP12	Human tissues	[72]
miR-186	Upregulated	AKAP12	Human tissues	[72]
miR-200	Upregulated	NRF2 pathway	Rat HCC model,	[73]
miR-210	Upregulated	VMP1	Human tissues, cultured cells	[74]
miR-216a	Upregulated	TSLC1	Human tissues, cultured cells	[75]
miR-216a/217	Upregulated	PTEN, SMAD7	Cultured cells, Human tissues	[76]
miR-221	Upregulated	CDK inhibitors	Transgenic mouse	[77]
	Upregulated	p27, p57, Arnt	Primary hepatocytes	[78]
	Upregulated	Bmf	Cultured cells, human tissues	[79]
	Upregulated	p27, p57	Cultured cells, human tissues	[80]
miR-221/222	Upregulated	p27, DDIT4	Human tissues, mouse model	[81]
miR-224	Upregulated		Human tissues	[82]
	Upregulated	Atg5, Smad4, autophagy	Human tissues, HBV X transgenic mice	[83]
	Upregulated	API-5	Cultured cells, human tissues	[84]
	Upregulated		Human tissues	[85]
	Upregulated	API-5	Human tissues	[86]
miR-423	Upregulated	p21/waf1	Human tissues, cultured cells	[87]
miR-485-3p	Upregulated	MAT1, LIN28B	Human tissues, xenograft model	[88]
miR-490-3p	Upregulated	ERCIC3	Human tissues, cultured cells	[89]
miR-494	Upregulated	MCC	Human tissue, mouse liver cancer model	[90]
miR-495	Upregulated	MAT1, LIN28B	Human tissues, xenograft model	[88]
miR-517a	Upregulated		Human tissues, cultured cells	[91]
miR-657	Upregulated	TLE1, NF-κB	Human tissues, cultured cells	[92]
miR-664	Upregulated	MAT1, LIN28B	Human tissues, xenograft model	[88]
miR-1323	Upregulated		Human tissues	[93]

Table 2 Downregulated miRNAs in hepatocarcinogenesis

miRNA	Expression levels	Targets	Main tested samples	References
let-7a	Downregulated	STAT3	Cultured cells	[94]
let-7c	Downregulated		Human tissues, cultured cells	[95]
let-7g	Downregulated	COL12A	Cultured cells, human tissues	[96]
miR-7	Downregulated	PIK3CD	Cultured cells, human tissues	[97]
miR-10a	Downregulated	EphA4	Cultured cells	[98]
miR-10b	Downregulated		Human tissues	[99]
miR-15a/16	Downregulated		Cultured cells	[100]
miR-21	Downregulated		Human tissues	[82]
miR-26a	Downregulated	IL-6	Human tissues, xenograft model	[101]
	Downregulated	CyclinD2, E2	Cultured cells, mouse model	[102]
miR-29	Downregulated	Bcl2, Mcl1	Human tissues, cultured cells	[103]
miR-29b	Downregulated	MMP-2	Human tissues, cultured cell	[104]
miR-29c	Downregulated	SIRT1	Cultured cells	[105]
miR-34a	Downregulated	CCL22	Human tissues, cultured cells	[106]
miR-99a	Downregulated	PLK1	Human tissues, cultured cells	[107]
	Downregulated	IGF-1R	Human tissues, cultured cells	[108]
miR-100	Downregulated	PLK1	Human tissues, cultured cells	[107]
miR-101	Downregulated	EZH2, EED	Human tissues, cultured cells	[109]
	Downregulated		Human tissues, cultured cells	[95]
	Downregulated	Mcl1	Cultured cells, human tissues	[110]
	Downregulated	Fos	Human tissues, cultured cells	[111]
miR-122	Downregulated	c-Myc	Human tissues, cultured cells	[112]
	Downregulated		Cultured cells	[113]
	Downregulated	MTTP	Knockout mice	[32]
	Downregulated	IL6, TNF	Knockout mice	[31]
	Downregulated	IGF-1R	Human tissues	[114]
	Downregulated	Cyclin G1	Human tissues, cultured cells	[115]
miR-124	Downregulated	ROCK2, EZH2	Human tissues, cultured cells	[116]
	Downregulated	CDK6, VIM, SMYD3, IQGAP1	Human tissues, cultured cells	[117]
miR-125a/125b	Downregulated		Human tissues, cultured cells	[118]
miR-125b	Downregulated	SUV39H	Human tissues, cultured cells	[119]
	Downregulated	Mcl1, Bclw, IL6R	Human tissues, cultured cells	[120]
	Downregulated		Human tissues, cultured cells	[95]
	Downregulated	PIGF, MMP-2, MMP-9	Human tissues, cultured cells	[121]
	Downregulated	Lin28B	Human tissues, cultured cells	[122]
miR-139	Downregulated	ROCK2	Human tissues, cultured cells	[123]
miR-139-5p	Downregulated		Human tissues, cultured cells	[95]
miR-140-5p	Downregulated	TGFBR1, FGF9	Human tissues, cultured cells	[124]
		DNMT1	Knockout mice	[125]
miR-141	Downregulated	DLC-1	Human tissues	[126]
miR-145	Downregulated		Human tissues	[60]
	Downregulated	IRS1, IRS2, IGF-1R, b-catenin	Human tissues, cultured cells	[127]
	Downregulated		Human tissues	[85]
miR-148a	Downregulated	c-Met	Human tissues, cultured cells	[128]
	Downregulated	HRIP	Mouse xenograft model, cultured cells	[129]
	Downregulated	e-cadherin	Human tissues, cultured cells	[130]
	Downregulated	c-Myc	Cultured cells	[131]
miR-152	Downregulated	DNMT1, GSTP1, CDH1	Human tissues	[132]

Table 2 continued

miRNA	Expression levels	Targets	Main tested samples	References
miR-195	Downregulated	NF-κB pathway	Cultured cells	[133]
	Downregulated	VEGF, VAV2, CDC42	Cultured cells, human tissues	[134]
	Downregulated	Cyclin D1, CDK6, E2F3	Cultured cells, human tissues	[135]
miR-198	Downregulated		Human tissues	[60]
miR-199a/b-3p	Downregulated	PAK4	Human tissues, cultured cells	[30]
miR-199b	Downregulated		Human tissues	[85]
miR-200a	Downregulated	H3 acetylation	Human tissues, cultured cells	[136]
miR-200b	Downregulated		Human tissues, cultured cells	[95]
miR-200c	Downregulated		Human tissues	[82]
miR-200	Downregulated		Human tissues	[82]
miR-203	Downregulated	ABCE1	Human tissues, cultured cells	[117]
miR-214	Downregulated	HDGF	Human tissues, cultured cells	[137]
miR-222	Downregulated		Human tissues	[82]
miR-223	Downregulated	STMN1	Human tissues	[138]
miR-224	Downregulated		Human tissues	[139]
miR-363-3p	Downregulated	c-Myc	Cultured cells	[131]
miR-375	Downregulated	ATG7	Human tissues, cultured cells	[140]
	Downregulated	AEG-1	Human tissues, cultured cells	[141]
miR-429	Downregulated	Rab18	Cultured cells	[142]
miR-449	Downregulated	c-MET	Xenograft, cultured cells	[143]
miR-520e	Downregulated	NIK	Human tissues, cultured cells	[69]
miR-612	Downregulated	AKT2	Cultured cells, human tissues	[144]
miR-637	Downregulated	STAT3 activation	Human tissues, cultured cells	[145]
miR-1271	Downregulated	GLP3	Human tissues, cultured cells	[99]

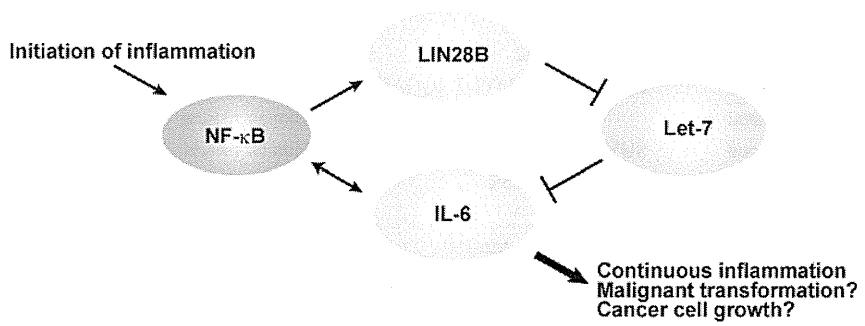

Fig. 2 A model bridging chronic inflammation and transformation by miRNA. Inflammation triggers activation of NF-κB, which leads to transcription of LIN28B. LIN28B inhibits the production of Let-7. Let-7 normally inhibits IL-6 expression, resulting in higher levels of IL-6 than are achieved by NF-κB activation. IL-6 mediated STAT3 activation is necessary for transformation and IL-6 activates NF-κB, completing a positive feedback loop

miRNAs, as a new class of gene expression regulators, may be involved in chronic inflammation-induced carcinogenesis and, in fact, several studies have clarified one such linkage, in which miRNAs may serve as a bridge between continuous inflammation and carcinogenesis.

A flagship report addresses a positive feedback loop of an inflammatory response mediated by NF-κB that activates Lin28B transcription (Fig. 2) [40]. LIN28B, which is an inhibitor of miRNA processing, reduces let-7 levels. Let-7 inhibits IL-6 expression, resulting in higher levels of IL-6 than achieved by NF-κB activation. IL-6-mediated STAT3 activation is necessary for transformation and IL-6 activates NF-κB, completing a positive feedback loop. Although the experiments mainly used MCF10A cells (breast cancer cells), a similar feedback loop was observed in HCC tissues. The authors termed these mechanisms an

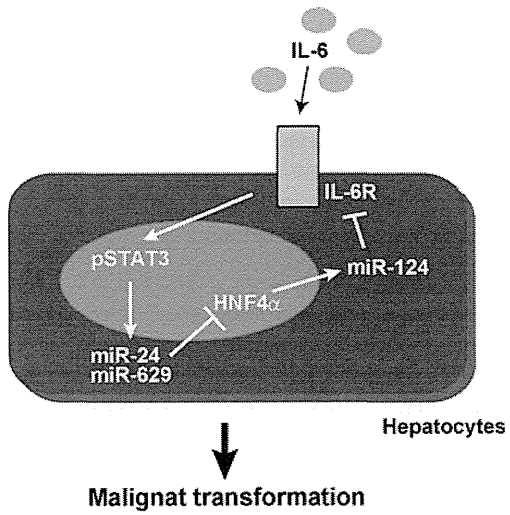

Fig. 3 A model describing a positive feedback loop mediated by miRNAs from transient HNF4α inhibition to transformation. Transient silencing of HNF4α is mediated by miR-24 and miR-629, both of which are induced by STAT3 activation following IL-6 stimulation. miR-124, whose promoter region contains HNF4α-binding sites, targets IL-6R and, thus, HNF4α silencing results in reduced expression of miR-124 and enhanced expression of IL-6R and activation of STAT3, which induces miR-24 and miR-629. This microRNA feedback-inflammatory loop is thought to be crucial in IL-6-mediated liver cancer

"epigenetic switch" because the loop maintains the epigenetic transformed state even in the absence of induction by inflammation (Fig. 2).

Another report addressed hepatocarcinogenesis induced by transient inhibition of HNF4α (Fig. 3) [41]. HNF4α was reported to be involved in liver oncogenesis, although discrepant reports have also been published [42–44]. In that report, transient HNF4α silencing was sufficient to maintain cell transformation. Through a miRNA library screen, miR-24 and miR-629 were identified to target HNF4α. Interestingly, both miRNAs were induced following HNF4α silencing, supporting their involvement in the HNF4α-dependent feedback loop. miR-24 and miR-629 contain the STAT3-binding motif in their promoter region. The authors showed that in response to IL-6, STAT3 binding to their promoters increased, resulting in miRNA expression. They also identified miR-124, whose promoter region contains HNF4α binding sites. miR-124 targets IL-6R and, thus, HNF4α silencing results in reduced expression of miR-124 and enhanced expression of IL-6R and activation of STAT3. The importance of these feedback loops was confirmed in vivo using a mouse HCC model induced by diethylnitrosamine. miR-124 delivery by cationic liposomes prevented tumor development. Thus, these microRNA feedback-inflammatory loops are important and can be a therapeutic target for liver cancer (Fig. 3) [41].

A recent paper reported a similar but distinct observation (Fig. 4). The authors found that when using DEN-induced foci of altered hepatocytes (FAH), LIN28-expressing cells are present in FAH, in which let-7 is down-regulated, resulting in the enhanced expression of IL-6, mediating the progression of malignancies from progenitors. An important difference between the cells in FAH and those in early hepatocarcinogenesis is that IL-6 signaling is autocrine, being mediated by reduced let-7 due to upregulation of LIN28B in FAH cells. This mechanism may contribute to malignant progression from HCC progenitor cells (Fig. 4) [45].

These three reports are from related research groups, and rely on the hypothesis that the IL-6-STAT3 pathway is crucial for hepatocarcinogenesis. Although IL-6 has been implicated as a growth factor in various epithelial cancers [46, 47], its relevance in hepatocarcinogenesis needs to be confirmed to determine the applicability and reproducibility of these findings to the clinical setting.

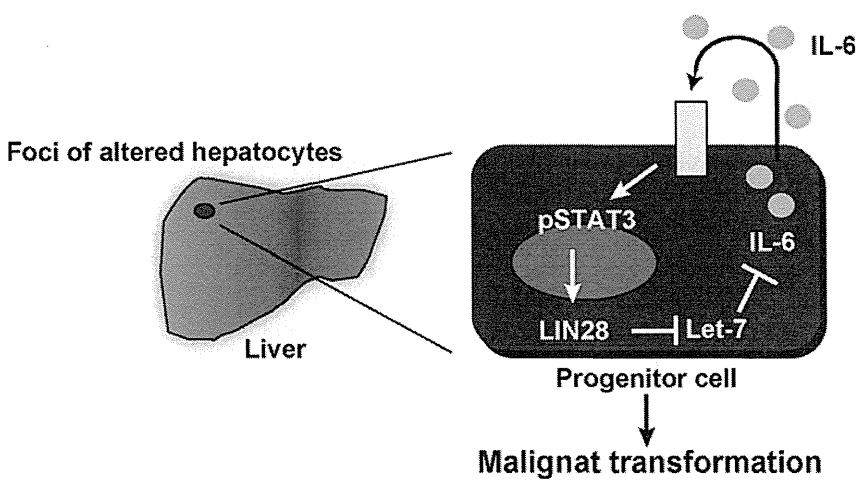

Fig. 4 A model bridging the malignant transformation of precursor cells and autocrine-mediated inflammation by microRNA. LIN28-expressing cells exist in the foci of altered hepatocytes, in which let-7 is downregulated, resulting in enhanced IL-6 expression, which mediates the progression of malignancies from progenitor cells

miRNAs as therapeutic targets in the liver

Recently, miravirsen, a LNA-modified DNA phosphorothioate antisense oligonucleotide against miR-122, became the first miRNA-targeting drug for clinical use [48]. It was developed to target HCV, as the stability and propagation of this virus is dependent on a functional interaction between the HCV genome and miR-122 [49, 50]. No harmful events were observed in Phase I studies in healthy volunteers, and Phase II studies proceeded to evaluate the safety and efficacy of miravirsen in 36 patients with chronic HCV genotype 1 infection. The patients were randomly assigned to receive 5 weeks of subcutaneous miravirsen injections at 3, 5 or 7 mg per kg body weight or a placebo over a 29-day period. Miravirsen resulted in a dose-dependent reduction in HCV levels, without major adverse events and with no escape mutations in the miR-122 binding sites of the HCV genome [48]. The success of miravirsen is promising, not only as a novel anti-HCV drug, but also as the first trial of miRNA-targeting therapy.

In addition to miravirsen, a clinical trial of MRX34 as a mimic of miR-34 is underway. MRX34 is a liposome-formulated mimic of the tumor suppressor miR-34 (Mirna Therapeutics, Austin, TX, USA). Further study of MRX34 is being conducted by Mirna Therapeutics, which initiated a Phase I study in May 2013 to examine the effects of MRX34 on unresectable primary liver cancer or advanced or metastatic cancer with liver involvement (ClinicalTrials.gov Identifier: NCT01829971). If these oligonucleotide therapies are successful, therapeutic options based on the numerous miRNAs deregulated during hepatocarcinogenesis appear promising [51].

Issues to be resolved in miRNA involvement in hepatocarcinogenesis

As described above, along with recent discoveries of the diverse effects of miRNAs in hepatocarcinogenesis, miRNA-mediated intervention is promising for the development of new diagnostic, preventive and therapeutic tools. However, the data obtained to date are far from complete. The following are some of the critical issues that we believe need to be resolved.

1. The reason for the non-reproducible results among studies should be determined to utilize the available data more reasonably and efficiently.
2. Identification of crucial driver miRNAs among the diverse deregulated miRNAs is critical to develop useful therapeutics in clinics, although even passive miRNAs may be utilized as markers for diagnosis or prediction of prognosis.
3. Comprehensive target gene analyses using in silico systems biology models should be applied.
4. For effective interventions using miRNA, the delivery method, improved oligonucleotide modification and safety must be further considered. Since miRNAs generally have diverse effects due to targeting multiple mRNAs, undesired outcomes, so called off-target effects, may be encountered, even when a specific miRNA is targeted.

Finding solutions to these issues should be considered as critically important for the near future in order to understand more fully the physiological function of miRNAs in hepatocarcinogenesis and utilize this knowledge in translational research.

Conclusions

The discovery of miRNA has, without doubt, opened up new possibilities for understanding the molecular mechanisms of gene regulation. As numerous findings regarding miRNA, from diverse perspectives, have been reported, the speed of discovery in this field is astonishing. In fact, novel therapeutics targeting miRNAs have already been successfully applied in clinical trials. Some miRNAs may be useful as novel biomarkers. Additionally, the discovery of novel concepts in the pathogenesis of hepatocarcinogenesis frequently involves miRNA. On the other hand, several important issues remain to be resolved in this field. Thus, continuous research in this field is still necessary to develop truly innovative concepts in our understanding of pathogenesis related to miRNA and to transform the obtained knowledge into real clinical applications.

Conflict of interest The authors declare that they have no conflict of interest.

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Original Article

Effect of the infectious dose and the presence of hepatitis C virus core gene on mouse intrahepatic CD8 T cells

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Aim: Chronic hepatitis C viral (HCV) infections often result in ineffective CD8 T-cell responses due to functional exhaustion of HCV-specific T cells. However, how persisting HCV impacts CD8 T-cell effector functions remains largely unknown. The aim of this study is to examine the effect of the infectious dose and the presence of HCV core gene.

Methods: We compared responses of intrahepatic CD8 T cells during infection of wild-type or HCV core transgenic (Tg) mice with various infectious doses of HCV-NS3-expressing recombinant adenovirus (Ad-HCV-NS3).

Results: Using major histocompatibility complex class I tetramer and intracellular interferon (IFN)-γ staining method to track HCV-NS3-specific CD8 T cells, we found that a significant expansion of HCV-NS3-specific CD8 T cells was restricted to a very narrow dosage range. IFN-γ production by intrahepatic CD8 T cells in HCV core Tg mice was suppressed as compared with wild-type mice. Higher levels of expression of regulatory molecules, Tim-3 and PD-1, by intrahepatic CD8 T cells and PD-L1 by intrahepatic antigen-presenting cells were observed in HCV core Tg mice following Ad-HCV-NS3 infection, and the expression increased dependent on infectious dose. Furthermore, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules in antigen-specific intrahepatic CD8 T cells.

Conclusion: High infectious dose and the presence of HCV core gene were strongly involved in ineffective CD8 T-cell responses. We consider that HCV core Tg mouse infected with high infectious dose of Ad-HCV-NS3 is useful as a chronic infection model in the development of immunotherapy for chronic hepatitis C.

Key words: core, functional exhaustion, hepatitis C, infectious dose, T cell

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a positive-sense single-stranded RNA virus of the genus *Hepacivirus* in the family *Flaviviridae*, and it infects 170 million people worldwide.[1] Approximately 10–60% of the patients clear HCV spontaneously during the acute phase of infection,[2] while the others develop chronic persistent HCV infection that eventually leads to liver cirrhosis and hepatocellular carcinoma.[3] HCV-specific cytotoxic T lymphocytes (CTL) play a major role in viral control during acute infection.[4] Nevertheless, during persistent infection, HCV-specific CTL effector functions are significantly impaired.

T-cell exhaustion is one of the remarkable features of chronic HCV infection. In chronically HCV-infected individuals, the frequencies of CTL are relatively low; similarly, the proliferative capacity as well as effector functions of HCV-specific T cells are impaired, and the production of type I cytokines (i.e. interleukin-2 and interferon [IFN]-γ) is dramatically suppressed.[5–8]

It appears that the major factors which determine duration and magnitude of an antiviral immune response are antigen (Ag) localization, dose and kinetics.[9] For example, high doses of widely disseminating strains of lymphocytic choriomeningitis virus (LCMV) exhaust antiviral CTL leading to establishment of a persistent infection.[10] Physical deletion of anti-LCMV CTL is most likely preceded by their functional impairment with the inability to produce effector cytokines.[11,12]

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Moreover, Wherry *et al.* showed that not only the persistence of a viral Ag, but also the initial Ag level is an important factor determining the quality of the antiviral memory response.[13]

Hepatitis C virus core protein has been reported to suppress T-cell response. HCV core-mediated inhibition of T-cell response can occur via either modulation of pro-inflammatory cytokine production by antigen-presenting cells (APC; i.e. monocyte and dendritic cells)[14] or direct effect on T cells.[15–17] Because the liver is the major site of HCV infection, it is crucial to understand the regulation of host immunity by HCV core in the liver compartment and the impact of HCV core-induced immune dysregulation in facilitating HCV persistence.

Hepatitis C virus does not infect small laboratory animals. The lack of a small animal model has hampered studies attempting to elucidate the mechanism of HCV-mediated suppression of antiviral CD8 T-cell activity and caused difficulty in the development of a therapeutic and/or prophylactic HCV vaccine.

Adenoviral vectors efficiently and reproducibly transfer foreign DNA into the livers of immunocompetent experimental animals. i.v. administration of adenoviral vectors of more than 10^9 infectious units/mouse results in infection and Ag expression in more than 90% of hepatocytes and acute self-limiting viral hepatitis.[18,19]

In this study, to develop a useful animal model in the development of immunotherapy for chronic hepatitis C, we examined the responses of intrahepatic CD8 T cells of HCV core transgenic (Tg) mice with various infectious doses of HCV-NS3-recombinant adenovirus (Ad-HCV-NS3).

METHODS

Mice

C57BL/6 MICE WERE purchased from Clea Japan (Tokyo, Japan), and Tokyo Laboratory Animal Science (Tokyo, Japan). Production of HCV core Tg mice has been described.[20] The core gene of HCV placed downstream of a transcriptional regulatory region from hepatitis B virus, which has been shown to allow an expression of genes in Tg mice without interfering with mouse development,[21] was introduced into C57BL/6 mouse embryos (Clea Japan). Eight- to 10-week-old mice were used for all experiments. The mice were housed in appropriate animal care facilities at Saitama Medical University (Saitama, Japan) and were handled according to international guidelines. The experimental protocols were approved by the Animal Research Committee of Saitama Medical University (#855).

HCV-NS3 recombinant adenovirus

Adenovirus HCV-NS3 expressing the fusion protein, comprising the entire HCV-NS3 and 3X flag, was constructed by using the AdEasy XL adenoviral vector system (Agilent Technologies, Santa Clara, CA, USA). The HCV-NS3 gene corresponding to amino acid residues 1027–1657 was amplified from the plasmid pBRTM/HCV1-3011con which contains the entire DNA sequence derived from the HCV H77 clone (kindly provided by Charls M. Rice, The Rockefeller University, New York, NY, USA)[22] by polymerase chain reaction. The recombinant Ad-HCV-NS3 vector was linearized by PacI digestion, and then transfected into 293 cells using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) to generate adenovirus. Ad-HCV-NS3 expressing transgene NS3 was amplified in 293 cells, purified by a series of cesium chloride ultracentrifugation gradients and stored at −80 °C until use. Mice were injected i.v. with 2×10^7, 1×10^9 and 1×10^{10} plaque-forming units (PFU) of Ad-HCV-NS3 or Adψ5 control vector. The experimental protocol regarding construction of recombinant adenovirus and infection of mice was approved by the Recombinant DNA Advisory Committee of Saitama Medical University (#1073).

Isolation of intrahepatic leukocytes

The liver was perfused with phosphate-buffered saline (PBS) plus 0.05% collagenase via the portal vein. Perfused livers were smashed through a 100-μm cell strainer (BD Biosciences, San Jose, CA, USA). The cell suspension was centrifuged with 35% Percoll at 320 *g* for 10 min, and the cell pellet was cultured in a plastic Petri dish in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; R-10) for 1.5 h to remove adherent cells. Then, non-adherent cells were harvested, washed twice with R-10 and used as intrahepatic lymphocytes (IHL). Adherent cells were used as intrahepatic APC).

Intracellular IFN-γ staining

The IHL were resuspended in R-10. In each well of a 96-well round-bottomed plate, 2×10^6 IHL were incubated for 5 h at 37 °C in R-10 containing 50 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MI, USA), 1 μM ionophore A23187 (Sigma-Aldrich) and 1 μg/mL brefeldin-A (BD Biosciences). The cells were then washed twice with ice-cold PBS (−) and incubated for 10 min at 4 °C with a rat antimouse

CD16/CD32 monoclonal Ab (mAb; Fc Block; BD Biosciences) at a concentration of 1 μg/well. Following incubation, the cells were washed twice with ice-cold PBS (−) and stained with a PE-conjugated HCV-NS3 H-2Db tetramer (Tet-603; GAVQNEVTL; Medical and Biological Laboratories, Nagoya, Japan)[23] and peridinin chlorophyll protein (PerCP)-conjugated rat antimouse CD8 MAb (clone 53-6.7; BD Biosciences) for 30 min at 4°C in staining buffer (PBS with 1% FCS and 0.1% NaN_3). After the cells were washed twice, they were fixed and permeabilized by using a Cytofix/Cytoperm kit (BD Biosciences) and stained with a fluorescein isothiocyanate (FITC)-conjugated rat antimouse IFN-γ mAb (clone XMG1.2; BD Biosciences). After the cells were washed, flow cytometric analyses were performed with a FACScanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and the data were analyzed with FACSdiva software (Becton Dickinson).

PD-1 and Tim-3 staining

Intrahepatic lymphocytes were prepared and treated with an antimouse CD16/CD32 mAb as described above for intracellular IFN-γ staining and then stained with a PE-conjugated HCV-NS3 H-2Db tetramer, PerCP-conjugated anti-CD8a (BD Biosciences), FITC-conjugated anti-PD-1 (eBioscience, San Diego, CA, USA) and Alexa647-conjugated anti-Tim-3 (Biolegend, San Diego, CA, USA) for 30 min at 4°C. After the cells were washed twice, they were fixed with PBS containing 1% formaldehyde and 2% FCS and analyzed by flow cytometry.

PD-L1 staining

Intrahepatic APC were prepared and treated with an antimouse CD16/CD32 mAb as described above for intracellular IFN-γ staining and then stained with a FITC-conjugated anti-CD11c (BD Biosciences) and PE-conjugated anti-PD-L1 (eBioscience) for 30 min at 4°C. After the cells were washed twice, they were fixed with PBS containing 1% formaldehyde and 2% FCS and analyzed by flow cytometry.

HCV core Ag detection

For the detection of HCV core Ag in the liver, liver tissue samples isolated 7 and 14 days post-infection were homogenized in RIPA B buffer (50 mM Tris pH 7.5, 1% NP40, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride) to make 10% (w/v) extract. Liver tissue extracts were assessed using Lumispot Eiken HCV Ag assay kit (Lumispot-Ag; Eiken Chemical, Tokyo, Japan).

Histology and immunohistology staining

Liver tissue samples isolated 7 and 14 days post-infection were used for histological studies. Paraffin sections (4-μm thick) were stained with hematoxylin–eosin safranin O. For immunohistology, 5-μm thick acetone-fixed frozen sections were incubated with rat anti-CD8 (BD Biosciences), followed by biotin-conjugated antirat immunoglobulin G and ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Persisting HCV-NS3 Ag detection

For the detection of persisting HCV-NS3 Ag in the liver, liver tissue samples isolated 21 days post-infection were homogenized in RIPA C buffer (50 mM Tris pH 7.5, 1% Triton X-100, 300 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.02% NaN_3) to make 2% (w/v) extract and used for immune precipitation/western blot assay. Liver tissue extracts were incubated with protein-G sepharose beads for 30 min at 4°C to remove non-specifically bound proteins. After centrifugation, supernatants were incubated with anti-Flag-M2 antibody (Sigma-Aldrich) coupled protein-G sepharose beads for 2 h at 4°C. After centrifugation, HCV-NS3-3xFlag fusion protein bound to the beads were dissolved in sample buffer and separated on 10% sodium dodecylsulfate polyacrylamide gel electrophoresis gels (Mini PROTEAN TGX gel; Bio-Rad, Hercules, CA, USA) for immunoblot analysis using anti-Flag-M2 antibody and goat anti-mouse Ig horseradish peroxidase (KPL, Gaithersburg, MD, USA). Electrochemiluminescence Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) was used for chemiluminescent detection.

Statistical analysis

Mann–Whitney *U*-tests were used to evaluate the significance of the differences. Correlations between parameters were tested for statistical significance by Pearson correlation.

RESULTS

Functional exhaustion of Ag-specific CD8 IHL with high infectious dose and the impaired Ag-specific CD8 IHL responses in core Tg mice

TO DETERMINE THE effect of the amount of virus dose, we evaluated hepatic inflammation and compared the magnitude of HCV-NS3-specific CD8

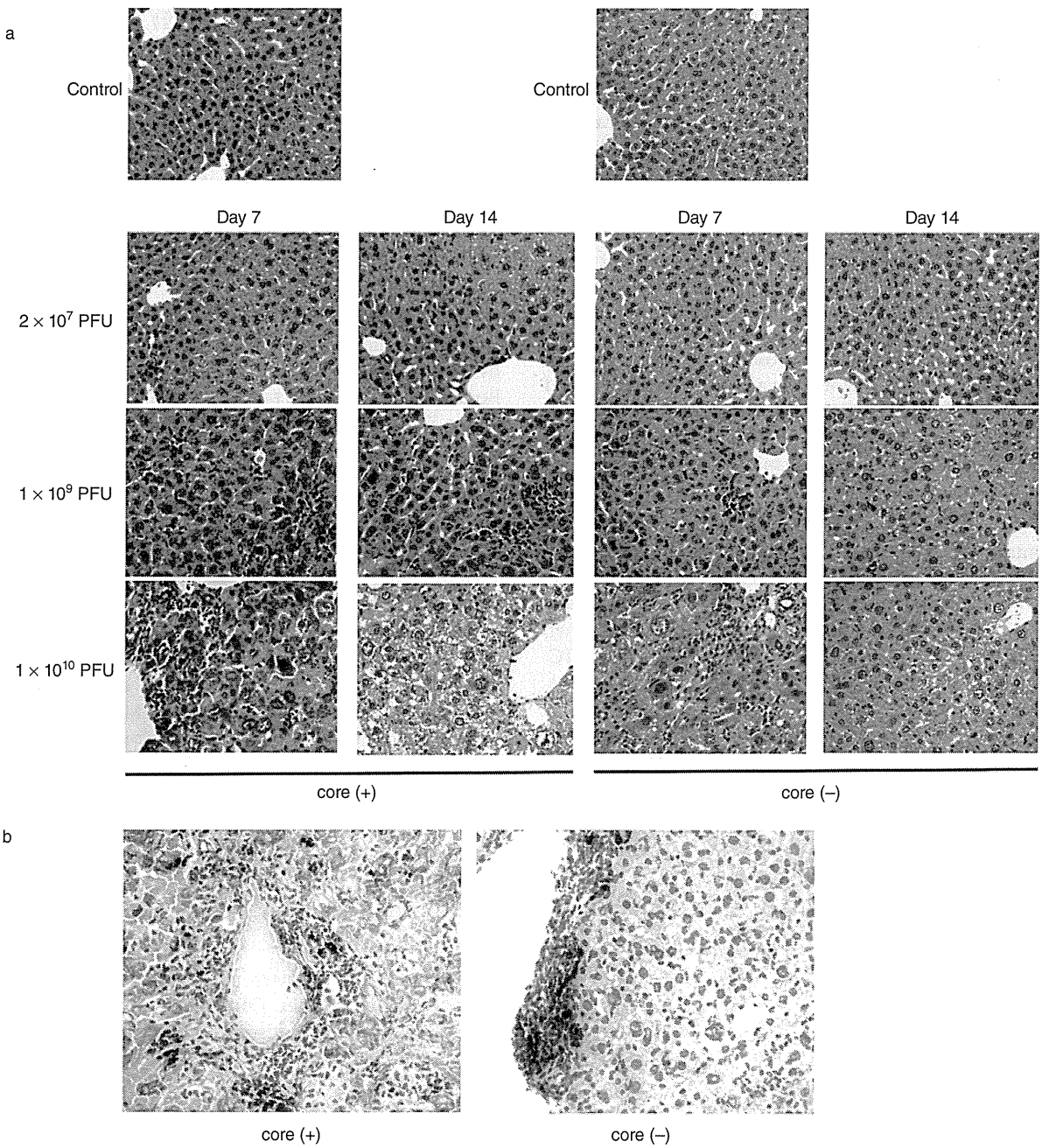

Figure 1 Adenovirus (Ad) infection-mediated hepatic inflammation in mouse liver. Hepatitis C virus (HCV) core (+) and core (–) mice were infected with 2×10^7, 1×10^9 and 1×10^{10} plaque-forming units (PFU) of Ad-HCV-NS3 i.v. and analyzed at 7 and 14 days post-infection. (a) Harvested liver tissues were analyzed by hematoxylin–eosin staining for assessment of hepatic inflammation. (b) Frozen liver sections were analyzed by CD8 staining. Liver infected with 1×10^{10} PFU and harvested at 7 days post-infection was used (original magnifications: [a] ×100; [b] ×200). (c,d) The frequency and number of hepatic CD8 lymphocytes were assessed by flow cytometric analysis. There were no differences in the frequency and number of hepatic CD8 lymphocytes between core (+) mice and core (–) mice. (e) Detection of HCV core antigen in the liver. Liver tissue extracts were assessed using Lumispot Eiken HCV Ag assay kit. There were no differences in core protein expression between Ad-infected and non-infected livers. ■, day 7; ▨, day 14; □, day 21.

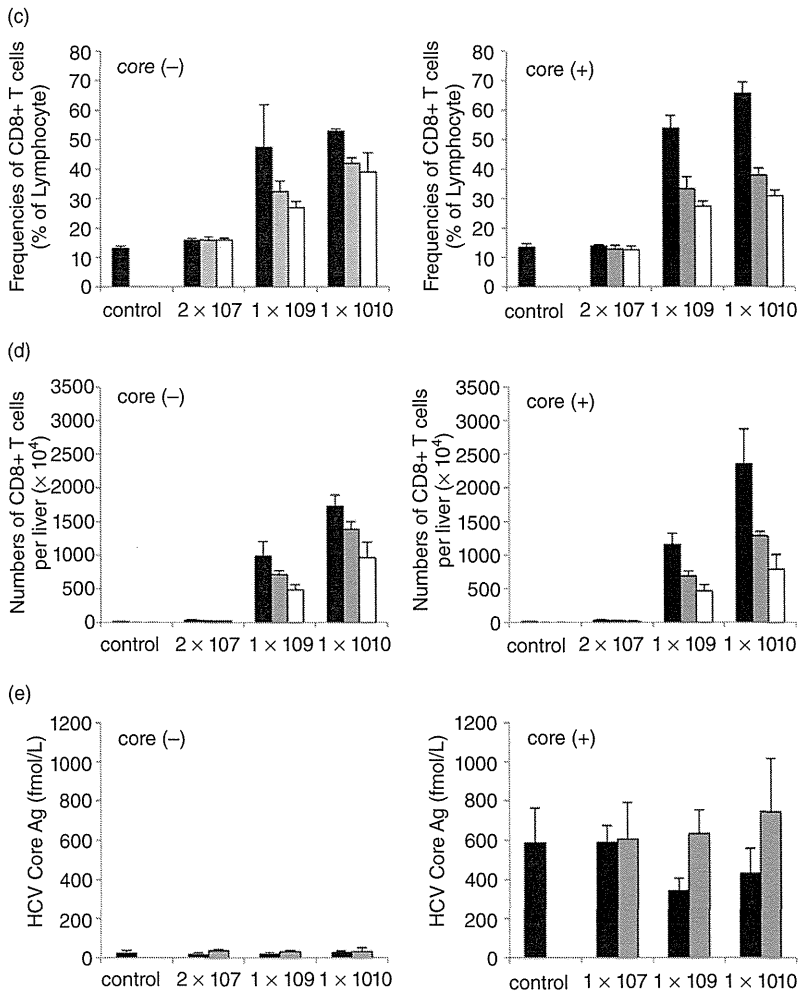

Figure 1 *Continued*

T-cell responses and their effector function in the liver of mice infected with 2×10^7, 1×10^9 and 1×10^{10} PFU Ad-HCV-NS3.

In histological studies, we observed Ad-infection-mediated hepatic inflammation in mice injected with 1×10^9 and 1×10^{10} PFU. Especially, infection with 1×10^{10} PFU caused drastic infiltrations of inflammatory cells (Fig. 1a). We also observed that CD8 lymphocytes infiltrated into the lobular areas of the infected liver in mice injected with 1×10^{10} PFU (Fig. 1b). At 7 days post-infection, we found by flow cytometric assay that the numbers and the frequencies of CD8 T cells in the liver were markedly increased after infection with 1×10^9 PFU and 1×10^{10} PFU, and the increased CD8 T cells decreased with time (Fig. 1c). We did not find significant differences between the number of CD8 T cells of core (+) and core (–) at each time point and infectious dose.

In addition, we evaluated core protein expression in the liver in each infectious dose at 7 and 14 days post-infection; there was no significant difference in core protein expression between Ad-infected and non-infected livers (Fig. 1e).

Using major histocompatibility complex (MHC) class I tetramer complexed with the H2-Db-binding HCV-NS3 GAVQNEVTL epitope, we found that i.v. infection with 2×10^7 PFU generally elicited only a weak expansion of HCV-NS3 tet^+ $CD8^+$ IHL (Fig. 2a,b) and IFN-γ^+ HCV-NS3 tet^+ $CD8^+$ IHL (Fig. 2a,c). In contrast, infection with 1×10^9 PFU induced a significant proliferation

of HCV-NS3 tet$^+$ CD8$^+$ IHL (Fig. 2a,b) and IFN-γ$^+$ HCV-NS3 tet$^+$ CD8$^+$ IHL (Fig. 2a,c).

In each infectious dose, HCV-NS3 tet$^-$ CD8 IHL did not show the diminution of elicited IFN-γ production (Fig. 2a). In contrast, HCV-NS3 tet$^+$ CD8 IHL showed the dose-dependent diminution of elicited IFN-γ production (Fig. 2d). Especially, infection with 1×10^{10} PFU led to a dramatic diminution of the elicited IFN-γ production in HCV-NS3 tet$^+$ CD8$^+$ IHL (Fig. 2a,d). These indicate that high infectious dose of Ad-HCV-NS3 cause NS3 Ag-specific immunosuppression.

As shown in Figure 2(c), the number of IFN-γ-producing HCV-NS3 tetramer$^+$ CD8 T cells in the liver of core (+) mice was lower than that of core (−) mice following PMA/ionophore stimulation. In addition, the percentage of IFN-γ-producing CD8 lymphocytes in tetramer$^+$ CD8 IHL of core (+) mice was suppressed as compared with core (−) mice following PMA/ionophore stimulation (Fig. 2d). These suggest that the presence of HCV core gene significantly impair antiviral effector CD8 T-cell responses in the liver.

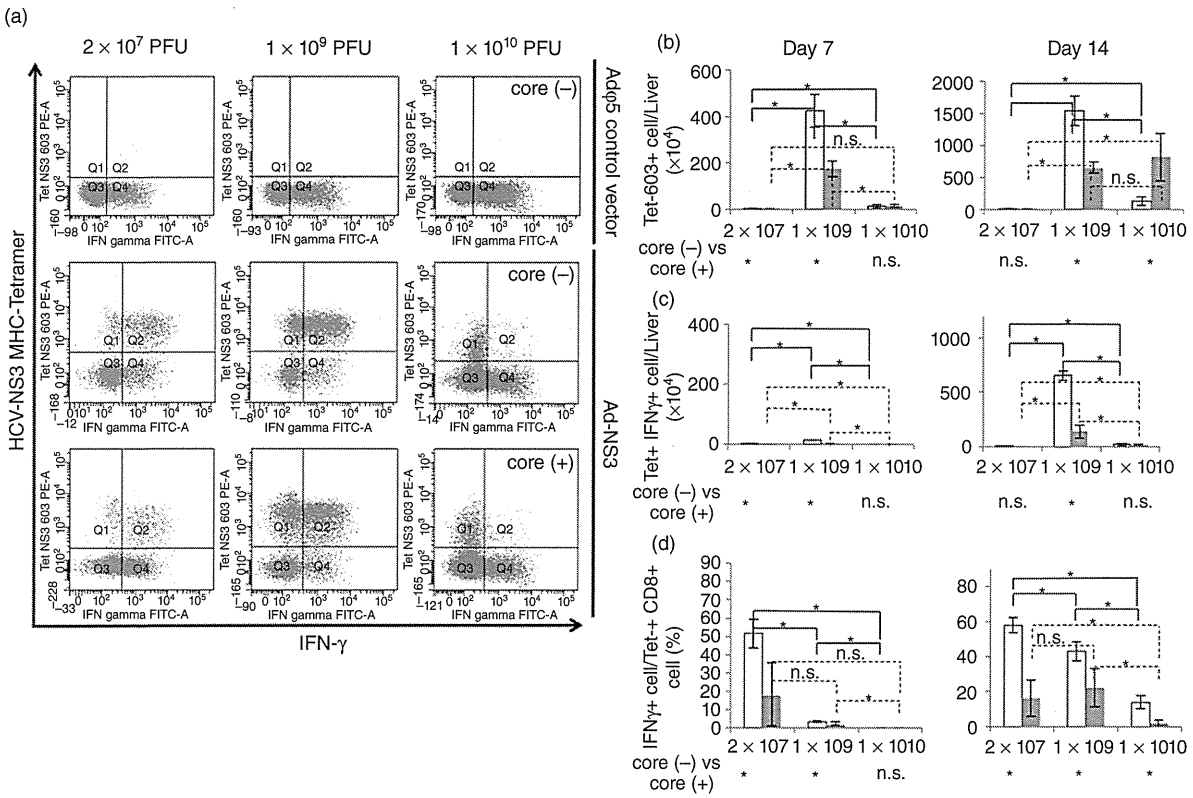

Figure 2 Impaired CD8$^+$ T-cell responses in the livers of high infectious doses. (a) Flow cytometric dot gram gating on the CD8 lymphocyte at day 14 post-infection. Graded doses of adenovirus (Ad)-hepatitis C virus (HCV)-NS3 were administrated i.v and NS3-specific intrahepatic cytotoxic T lymphocytes (CTL) were analyzed using major histocompatibility complex (MHC) class I tetramer and intracellular interferon (IFN)-γ staining method. Data show one representative mouse per group ($n = 3$). (b) The number of MHC tetramer$^+$ CD8 lymphocytes in the liver of core (−) and core (+) mice at day 7 and day 14 following Ad-HCV-NS3 infection (*$P < 0.05$; n.s., not statistically significant). (c) The number of tetramer+ intracellular IFN-γ + CD8 lymphocytes in the liver of core (−) and core (+) mice at day 7 and day 14 following Ad-HCV-NS3 infection. Intrahepatic lymphocytes (IHL) were restimulated with phorbol myristate acetate (PMA)/ionophore for 5 h and IFN-γ production was determined by intracellular cytokine staining (*$P < 0.05$; n.s., not statistically significant). (d) The percentage of intracellular IFN-γ$^+$ CD8 lymphocytes in tetramer$^+$ CD8 IHL of core (−) and core (+) mice on day 7 and day 14 following Ad-HCV-NS3 infection. IHL were restimulated with PMA/ionophore for 5 h and IFN-γ production was determined by intracellular cytokine staining (*$P < 0.05$; n.s., not statistically significant). □, core (−); ▩, core (+).

The existence of HCV core gene cause higher expression of suppression molecules

The PD-1 and Tim-3 inhibitory pathways have been reported to play important roles in the dysfunction of effector T-cell response during viral infection. For instance, the expression of PD-1 is increased on functionally exhausted CD8 T cells during chronic viral infection.[15] To investigate the relation between the viral infectious doses or the expression of HCV core gene in the liver and suppression marker expression of antiviral CD8 IHL, we examined the expression for both PD-1 and Tim-3 in the CD8 IHL and PD-L1 in the intrahepatic APC of core (+) and core (−) following various doses Ad-HCV-NS3 infection.

We found that i.v. infection with 1×10^{10} PFU induced a significant expression of PD-1 and Tim-3 by Ad-HCV-NS3 specific intrahepatic CD8 T cells (Fig. 3).

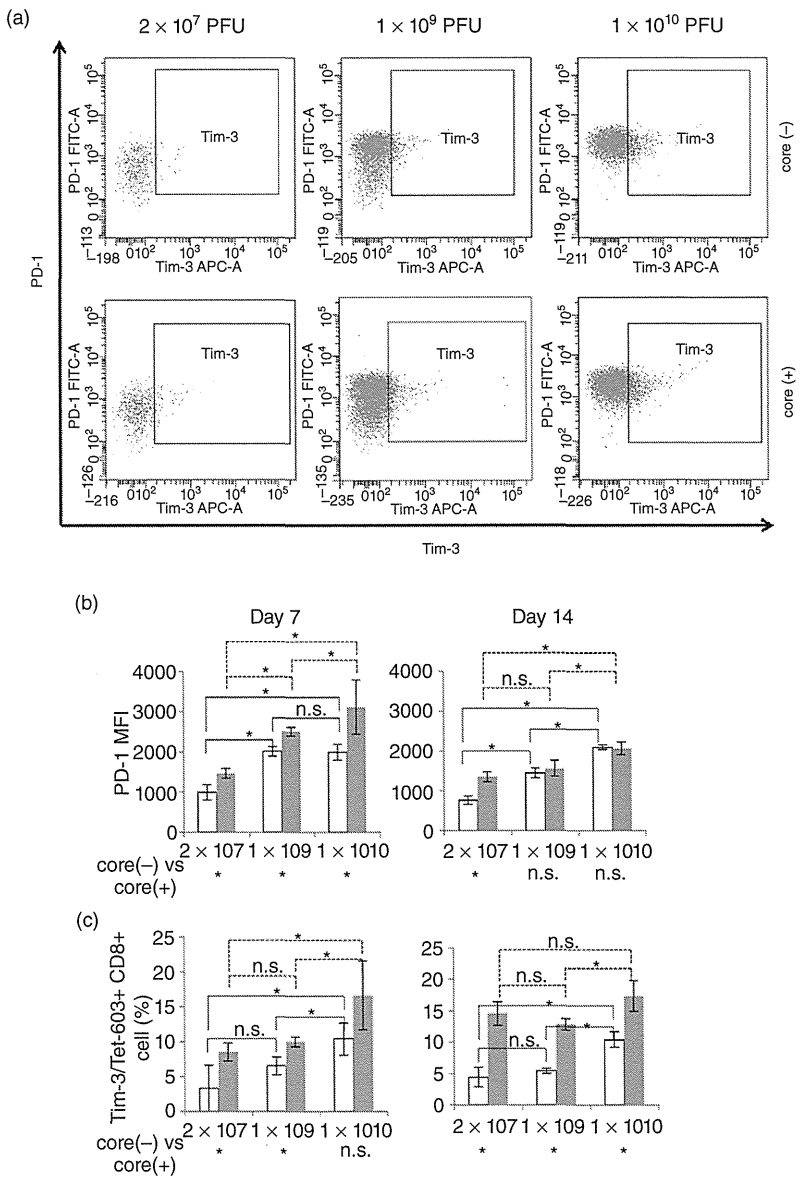

Figure 3 Differential suppression marker expression on NS3-specific CD8 lymphocyte in various infectious doses. (a) Flow cytometric dot gram gating on the hepatitis C virus (HCV)-NS3-tetramer$^+$ CD8 lymphocyte at day 14 post-infection. Graded doses of adenovirus (Ad)-HCV-NS3 were administrated i.v and NS3-specific intrahepatic cytotoxic T lymphocytes (CTL) were analyzed using major histocompatibility complex (MHC) class I tetramer and anti-PD-1 and anti-Tim-3 monoclonal antibody. Data show one representative mouse per group ($n = 3$). (b) The median fluorescence index (MFI) value of PD-1 expressed on HCV-NS3-specific CD8 intrahepatic lymphocytes (IHL) from core (−) and core (+) mice at 14 days following Ad-HCV-NS3 infection. ($^*P < 0.05$; n.s., not statistically significant). (c) The number of Tim-3$^+$ HCV-NS3-specific CD8 IHL from core (−) and core (+) mice at 14 days following Ad-HCV-NS3 infection. ($^*P < 0.05$; n.s., not statistically significant). □, core (−); ▩, core (+).

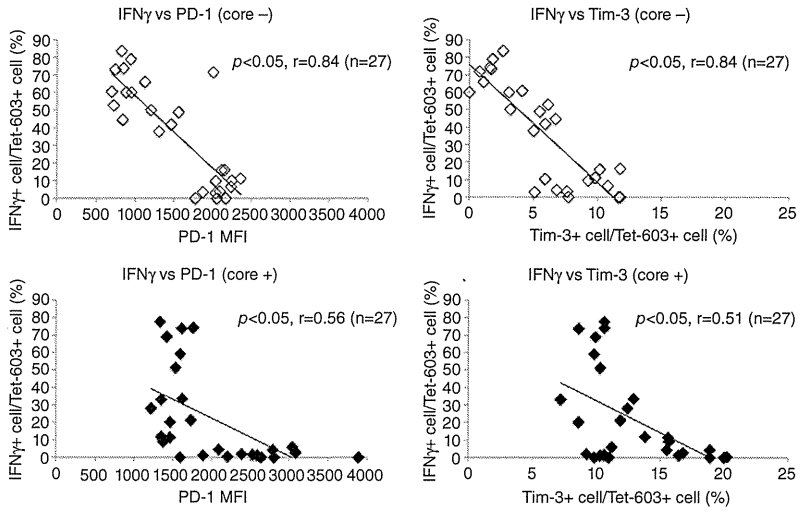

Figure 4 Inverse correlation between the percentages of interferon (IFN)-γ-producing cells and expression of regulatory molecules in antigen-specific intrahepatic CD8 T cells.

When core (+) and core (−) mice were compared, the expression of PD-1 and Tim-3 by Ad-HCV-NS3-specific intrahepatic CD8 T cells was significantly higher in core (+) than core (−) at various time points following Ad-HCV-NS3 infection. Furthermore, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules in Ag-specific intrahepatic CD8 T cells (Fig. 4).

To determine whether suppression ligand expression by intrahepatic APC is altered in core (+) mice, the intensity of PD-L1 expressed by $CD11^+$ cells was analyzed at 7 and 14 days post-infection. Intrahepatic APC showed the infectious dose-dependent augmentation of PD-L1 expression. We observed elevated expression of PD-L1 by APC in core (+) mice infected with 10^{10} PFU at both time points (Fig. 5a,b). In PD-L1 expression, we did not find a significant difference between Ad-HCV-NS3 infection and Adψ5 control vector infection (Fig. 5c,d).

Taken together, these data suggest that the existence of HCV core gene suppress T-cell-mediated immune response by causing higher expression of suppression molecules.

Ag persistence after Ad-HCV-NS3 infection

To determine the Ag persistence after Ad-HCV-NS3 infection, we analyzed the expression of FLAG-tagged HCV-NS3 protein in the liver by IP-western blot after administration of 2×10^7, 1×10^9 or 1×10^{10} PFU of the virus. The Ag expression in the liver could be found in both core (+) and core (−) mice on 21 days after infection with 1×10^{10} PFU. When 1×10^9 PFU of Ad-HCV-NS3 was administrated, HCV NS3-protein was almost cleared from the liver of core (−) mice at day 21 post-infection, whereas the Ag expression persisted in the liver of core (+) mice until day 21 post-infection (Fig. 6).

It is important to note that the loss of Ag expression in the liver of core (−) mice after infection with 1×10^9 PFU coincided with the high HCV-NS3-specific CD8 T-cell response at 14 days post-infection (Fig. 2c), whereas Ag persistence in the liver of core (+) mice after infection with 1×10^9 PFU or the liver of core (−) and core (+) mice after infection with 1×10^{10} PFU was associated with strongly diminished Ag-specific CD8 T-cell response (Fig. 2c). It is likely that the expression of core protein and the high amount of Ag in the liver contributed to the functional exhaustion of HCV-NS3-specific CD8 T cells.

DISCUSSION

IN THIS STUDY, we found an impaired response of HCV-NS3-specific intrahepatic CD8 T cell in a high dose setting (1×10^{10} PFU) of Ad-HCV-NS3 infection. Furthermore, higher levels of expression of regulatory molecules, Tim-3 and PD-1, by intrahepatic CD8 T cells and PD-L1 by intrahepatic APC were observed in HCV core Tg mice and the expression increased dependent on infectious dose. In addition, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules