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MINIREVIEWS

Heterogeneity of immune control in chronic hepatitis B virus infection: Clinical implications on immunity with interferon-α treatment and retreatment

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Abstract

Hepatitis B virus (HBV) infection is a global public health issue. Interferon-α (IFN-α) treatment has been used to treat hepatitis B for over 20 years, but fewer than 5% of Asians receiving IFN-α treatment achieve functional cure. Thus, IFN-α retreatment has been introduced to enhance antiviral function. In recent years, immune-related studies have found that the complex interactions between immune cells and cytokines could modulate immune response networks, including both innate and adaptive immunity, triggering immune responses that control HBV replication. However, heterogeneity of the immune system to control HBV infection, particularly HBV-specific $CD8^+$ T cell heterogeneity, has consequential effects on T cell-based immunotherapy for treating HBV infection. Altogether, the host's genetic variants, negative-feedback regulators and HBV components affecting the immune system's ability to control HBV. In this study, we reviewed the literature on potential immune mechanisms affecting the immune control of HBV and the clinical effects of IFN-α treatment and retreatment.

Key Words: Hepatitis B virus; Chronic; Functional cure; Heterogeneity; Immunity; Immune control; Interferon-α; Retreatment

Core Tip: Hepatitis B virus (HBV)-specific immune control is characterized by distinct phenotypical and functional profiles. Owing to the negative feedback associated with all immune responses in an infected host, immunomodulators regulating a single immune pathway are unlikely to fully restore antiviral immunity. Interferon-α (IFN-α) treatment was shown to simultaneously affect multiple immune pathways and various immune cell populations in the host and integrate signals toward improving HBV-specific immune control. In addition, IFN-α retreatment was shown to improved functional cure rates, indicating that could gradually enhance the overall immune control.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem. It is estimated that over 257 million people are suffering from chronic hepatitis B (CHB) infection[1]. After being infected by HBV, the virus transfers its genome into the nucleus of hepatocytes, where it is converted into covalently closed circular DNA (cccDNA), which has two major roles: Acting as a template for virus replication and acting as a reservoir for long-term virus preservation. Some studies have found that cccDNA persists in the hepatocytes of patients even decades after HBV infection has been resolved[2]. Further, it was shown that part of the viral genome remained integrated into the genomic DNA of the host's hepatocytes. Therefore, targeted eradication of cccDNA and viral genome in hepatocytes is regarded as the holy grail for curing HBV[3-5], which has not yet been achieved with current therapies.

HBV infection induces various immune responses that lead to heterogeneous immune control associated with the HBV infection[5,6]. Acute HBV infection can be terminated by the host's adaptive immune responses, characterized by multi-specific and vigorous HBV-specific $CD8^+$ T cell responses. In contrast, during CHB infection, the adaptive immune responses are severely depressed due to exhausted or reduced HBV-specific $CD8^+$ T-cells[6,7] and dysfunctions in HBV-specific B cells[7-9]. Researchers have noticed different outcomes from HBV infection in different populations and races.

Most CHB cases in the Asian population occur during infancy or childhood. However, asymptomatic infection during childhood makes it very challenging for authorities to determine when the infection actually occurred. Further, due to the uncertain timing of hepatitis flares and disease phases, there is little research on this topic in past literature, and little is known about hepatitis flares and intrahepatic immunity. Recently, studies on the partial immune mechanism in CHB have been performed, and important discoveries were made[2-6].

Currently, CHB is treated with nucleoside/nucleotide analogs (NAs) or interferon-α (IFN-α). NAs can target HBV polymerase/reverse transcriptase, inhibit HBV replication and are better tolerated by patients, but they cannot target cccDNA and unavoidably often results in NAs resistance and associated mutations. Since NAs do not directly influence immune response, functional cure with NAs is rarely achieved. In contrast, IFN-α treatment enhances HBV-specific immune control and can result in a partial or functional cure. However, due to poor efficacy with single course IFN-α treatment, since 1996, researchers have begun using IFN-α retreatment to improve the antiviral efficacy in CHB patients[10-15] or sufferers from NAs multi-drugs resistance[15-18].

In this present article, we reviewed the complex interactions between immune cells and cytokines of the immune response network against HBV, the correlation between host genetic variations and hepatitis B, the interplay between HBV components and HBV-specific immune control, and the heterogeneity of HBV-specifical immune control. Based on this foundation, we also discussed the underlying mechanism of IFN-α treatment and retreatment for improved HBV-specific immune control.

HETEROGENEOUS IMMUNITIES DURING HBV INFECTION

The innate and adaptive immunity work together to control immune responses against HBV. Innate immunity is not HBV antigen-specific but still produces T-cell polarizing and inflammatory cytokines that alter the intrahepatic microenvironment for presenting HBV antigens to naïve T cells to establish HBV-specific immunity. In recent years, researchers have recognized adaptive immunity as a crucial player for persistent and efficient immune control of HBV infection, which comprises a complex web of effector cell types. HBV-specific T cells help clearing HBV-infected hepatocytes and reduce the levels of circulating virus, while B cells neutralize viral particles and prevent reinfection[3,19-22]. Thus, their

levels ultimately determine the outcome of the disease.

The liver is an immunologically tolerant organ in which most immune cells are suppressed to limit hypersensitivity of immune responses against organ damage and local antigens. Thus, in a healthy state, the proportion of immune cells within the liver is much lower than in the peripheral blood. The difference in immune statuses between the liver and peripheral blood is defined as "immune compartmentalization". Investigations into the effects of costimulation have shown that Toll-like receptors (TLRs) and Treg cells participate in the intrahepatic immunopathogenesis in patients with HBV infection[3,23-25].

Recognition of HBV components by the innate immunity

Non-specific recognition of HBV components occurs at the molecular/subcellular level by innate immunosensors, namely pathogen-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMP). The main PRRs that sense viral infection consists of retinoic acid-inducible gene I (RIG-I)-like receptors, nucleotide-binding oligomerization domain-containing protein-like receptors, TLRs, DNA-sensing receptors and C-type Lectin. These PRRs are expressed in epithelial cells, endothelial cells and immune cells. When PRRs interact with their cognate PAMP, downstream signaling pathways cascade, including adaptor/co-adaptor molecules, kinases and transcription factors are activated. This leads to the expression of interferon-stimulated genes (ISGs) and NFκB-inducible or pro-inflammatory genes, and various inflammatory cytokines are secreted. Primary cytokines include various classes of IFNs, pro-inflammatory cytokines and chemokines. These cytokines recruit various immune cells to the HBV-infected site, causing direct or indirect antiviral actions. These immune responses induce T cell proliferation and increase the efficiency of HBV-specific CD8+ T cells[21,26,27]. In particular, TLRs in hepatocytes and hepatic non-parenchymal cells (NPCs) have been shown to play vital roles in antiviral immunity. Currently, the use of TLR agonists as therapeutic agents to treat CHB is being validated[7].

In addition to cytokines, innate effector cells also participate in the control of HBV infection. Innate effector cells, including natural killer (NK) cells, γδT cells and mucosal-associated invariant T cells, can restrain the virus but are not specific for controlling HBV. Activated NK cells can induce inflammation in the liver, while activated T cells can be killed by NK cells, thereby reducing HBV-specific T cells. Although NK cells can suppress HBV replication, their activity was shown to be inhibited by transforming growth factor β (TGF-β) and IL-10 in CHB patients[3]. In addition, hepatic PCs, such as KCs and liver sinusoidal endothelial cells, can stimulate innate and adaptive immunity against HBV infection[28,29].

In short, the activation of innate immunity can lead to the production of cytokines, enhance antigen presentation, alter the intrahepatic microenvironment and trigger adaptive immunity (Figure 1).

HBV infection and adaptive immunity

HBV-specific T cells play a vital role in controlling HBV infection, and their immune responses can lead to the resolution of HBV replication. In acute hepatitis B patients, spontaneous viral clearance can occur *via* multi-specific $CD8^+$ T cell responses against HBV components[3,30]. In contrast, in CHB, the patients suffer from the exhaustion of HBV-specific $CD8^+$ T-cell responses throughout the HBV infection. Thus, most research on immune dysfunction focus on the activity of T cells, including $CD8^+$ and $CD4^+$ T-cells.

CHB is associated with the exhaustion of HBV-specific $CD8^+$ T-cells, marked by compromising functionality, such as reduced production of antiviral cytokines and immunodulatory cytokines and impaired proliferative capacities, defined as T-cell exhaustion[31,32]. HBV-specific $CD8^+$ T-cell exhaustion can be induced by: (1) Gradual aggravation of $CD8^+$ T-cells dysfunction and decreasing inflammatory cytokines production; (2) Increase in checkpoint inhibitors and immunosuppressive cytokines; (3) Epigenetic alterations leading to unrecoverable $CD8^+$ T-cell functions; (4) Alterations in $CD8^+$ T-cell phenotype; (5) Mitochondrial dysfunction and glycolysis downregulation, (6) Decrease in cell detection rate; and (7) Terminal exhaustion and physical deletion of HBV-specific $CD8^+$ T-cells.

In acute hepatitis B, $CD4^+$ T-cells have an important but indirect role in cleaning the virus. Th1-polarized $CD4^+$ T-cells regulate and maintain $CD8^+$ T-cell responses, contributing to HBV clearance. In contrast, during chronic HBV infection, the activation and upregulation of $CD4^+CD25^+$ Treg-cells suppress effective antiviral immune responses by inhibiting IFN-γ secretion, which inhibits the proliferation and cytokine secretion of $CD4^+$ and $CD8^+$ T-cells[7,30]. Figure 1 illustrates the complex interactions between immune cells and cytokine within the immune response network. The immune cells and their related cytokines are presented in Table 1.

HBV components inhibit innate and acquired immunity

HBV is believed to have existed in the human population for thousands of years and evolved with humans. During this evolution process, HBV has developed a particular lifecycle based on its unique replication mode through cccDNA and viral components that can adapt and suppress its host's immunity. Although the innate immunity can differentiate between different HBV components, HBV virion, antigens and peptides are still able to attack the TLR signaling pathway, resulting in negligible ISGs or IFNs secretions[4,7].

Table 1 Immune cells and their corresponding cytokines released

Immune cells	Released cytokines
NK cells	IFN-γ, TNF-α, IL-10
NKT cells	IFN-γ, IL-4, IL-13
Monocytes	IFN-γ, TNF-α, IL-10
Macrophages	IFN-γ, IL-6, MCP-1, IL-1β, TNF-α, CXCL10
γδT cells	IL-17, TNF-α
HSCs	IL-1β, IL-6, TGF-β
Kupffer cells	IL-10, TGF-β, IL-12, IL-6, TNF-α
$CD8^+$ T cells	IFN-γ, TNF-α, IL-2, IL-10, IL-17, IL-21
CD4+ T cells	IFN-γ, IL-21,IL-17
Regulatory T cells	IL-10, TGF-β, IL-35
Regulatory B cells	IL-10

HSCs: Hepatic stellate cells; IL: Interleukin; TGF-β: Transforming growth factor β; TNF-α: Tumor necrosis factor-α; IFN-γ: Interferon-γ; NK: Natural killer.

Among the ISGs is the apolipoprotein B editing complex (APOBEC) gene. APOBEC3A/B has been shown to cause cccDNA degradation, while APOBEC3G can inhibit HBV replication. However, APOBEC3G expression is often reduced by HBsAg[32].

A viral protein known for influencing HBV replication is HBx. It can interact with the cellular proteins in hepatocytes to increase viral replication by impairing IFN signaling and enhancing HBV gene expression[33-35]. Further, it was shown that HBV antigens could inhibit $CD8^+$ T-cell efficiency. Antagonist functions may provide a means for HBV to escape immune detection. Considering that certain $CD8^+$ T-cell epitopes in hepatitis B core antigen (HBcAg) or HBsAg can act as T-cell receptor (TCR) antagonists, their binding to TCR can lead to the suppression of $CD8^+$ T-cell response, thus decreasing their efficiency (Figure 2). In addition, the chronicity of HBV infection was shown to be promoted by HBeAg *via* the induction of $CD8^+$ T-cell tolerance[36].

Mechanisms of immune escape in antigen-presenting cells/HBV specific-$CD8^+$ T-cells

When the HBV protein is swallowed by antigen-presenting cells (APCs), this protein is digested into tiny pieces, which are transferred onto human leukocyte antigen (HLA) antigens. The HLA antigen complex on APCs is then displayed to T cells, which produce effector molecules to eliminate HBV. However, when HBV enters a hepatocyte, it enhances intracellular survival at the expense of higher levels of HBV replication. Thus, HBV-infected hepatocytes produce a large number of HBV antigens that can, in turn, inhibit HBV antigen processing and presentation in APCs. Further, amino acids flanking the viral epitopes in APCs play a critical role in antigen processing. Mutations at these regions in HBV infection harm the proteasomal processing of epitopes and lead to $CD8^+$ T-cell escape[30].

HLA genes are critical for the immune system as they control pathogens and clear infections. Host HLA polymorphisms have been demonstrated to influence disease progression in HBV infection[37]. Studies found that changes in the surface expression of HLA class I complexes on APCs were associated with HBV replication and persistence. Lower HLA class I expression led to early HBeAg seroconversion, while down-regulation of HLA class II molecules led to pre-core mutants of HBcAg[38]. Lumley *et al*[30] focused on the interplay between the immune escape of HBV and the selective mutation of HLA-binding residues. They reported that this selective mutation of HLA-binding residues in $CD8^+$ epitopes could induce the immune escape of HBV, which is one of the most commonly identified mechanisms for HBV-specific $CD8^+$ immune escape[30].

N-linked glycosylation (NLG) is a post-translational modification that can impact the infectivity and antigenicity of HBV. It can mask immune epitopes, leading to immune escape and interfering with the antibody recognition of hepatitis B surface antigen. NLG can also affect the ability through which the envelope protein of HBV interacts with the surface of capsids to drive HBV virion secretion[30].

The connection between TCR on T-cell and HLA class I/peptide complexes induces the activation of $CD8^+$ T-cells, but alterations in TCR recognition, *i.e.*, epitope mutations in TCR contact residues, can lead to the immune escape of $CD8^+$ T-cells. Immunodominance of HBV epitopes is ensured by the amino acid sequence of the peptide and its concentration and binding affinity with T cell clones. In different $CD8^+$ T-cell clones, the same viral peptide can induce different signaling cascades[22]. Figure 2 illustrates the immune escape of HBV, which can occur through multiple pathways. This has a vital role in HBV infection that can last for decades, with some T cell defects being irreversible.

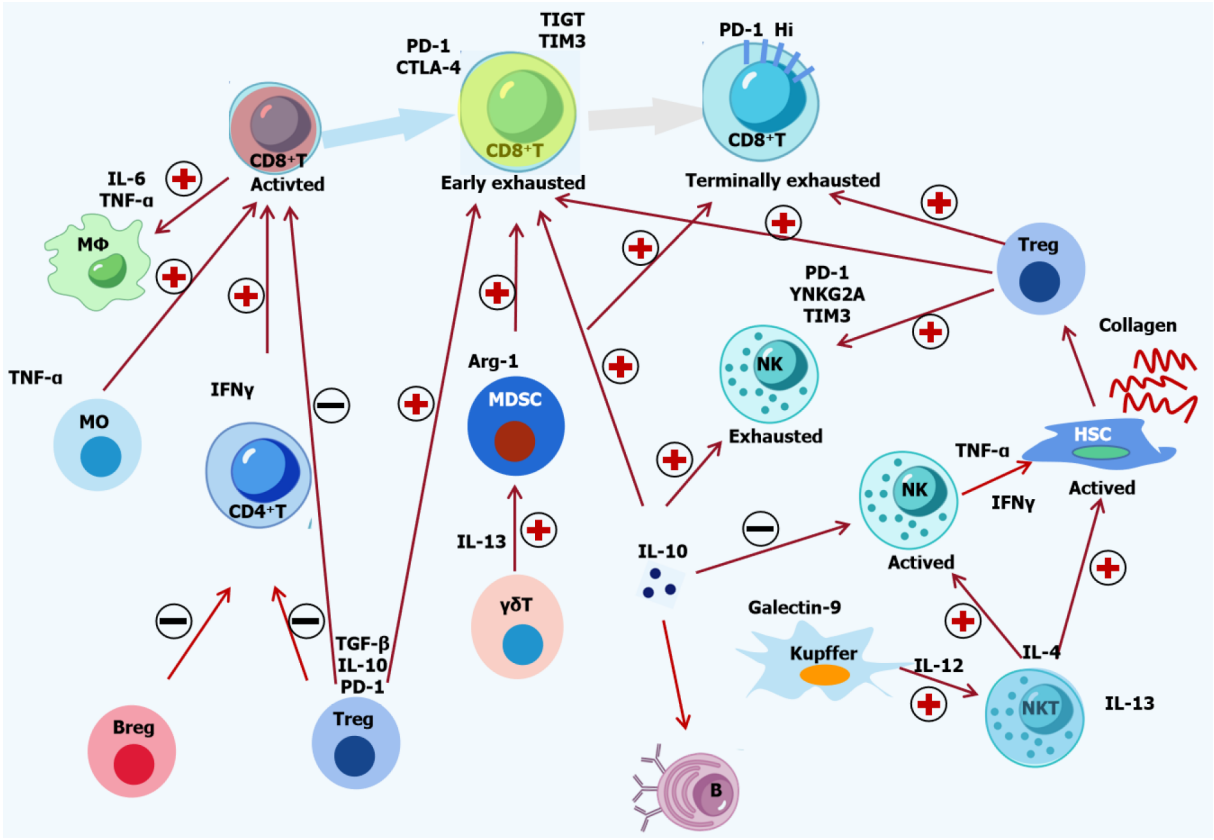

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Figure 1 Crosstalk among immune cells and cytokines in hepatitis B virus infection. The complex interactions among immune cells and cytokines in chronic hepatitis B are shown. Hepatitis B virus (HBV)-specific CD8$^+$ T-cells are activated by monocytes and CD4$^+$ T-cells, followed by recruitment and activation of macrophages by active CD8$^+$ T-cells. The activation of natural killer (NK) T-cells is induced by Kupffer cells, which activate NK cells and hepatic stellate cells (HSCs). Suppressive Tregs, Bregs and Kupffer cells induce the functional impairment of CD8$^+$ T cells, CD4$^+$ T-cells and NK cells. Moreover, Treg cells, Kupffer cells and myeloid-derived suppressor cells can lead to the exhaustion of CD8$^+$ T-cells and NK cells. Inflammatory and inhibitory cytokines, including Monocyte chemoattractant protein-1, tumor necrosis factor-α, interferon-γ, interleukin (IL)-4, IL-6, IL-12, IL-13, IL-17, IL-10, and transforming growth factor-β, are involved in the crosstalk among immune cells. The activation of HSCs in sinusoids is induced by a complement protein such as C5a. Finally, decreasing epigenetic modification and function of HBV-specific CD8$^+$ T-cells inhibits the immune control of HBV. PD-1: Programmed death 1; CTLA-4: Cytotoxic T-lymphocyte antigen-4; IL: Interleukin; TNF-α: Tumor necrosis factor-α; TGF-β: Transforming growth factor β; IFN-γ: Interferon-γ; HSC: Hepatic stellate cell; MDSC: Myeloid-derived suppressor cells; NK: Natural killer.

Negative feedback regulation in immune response pathways

Negative feedback regulations commonly occur in immune response pathways. These regulations include negative regulation of immune signal pathways (*i.e.*, negative feedback regulation in TLRs pathway), activation of immune checkpoints, expression of inhibitory cytokines, and activation of inhibitory immune cells. An active innate immunity can induce the secretion of cytokines with antiviral activity, enhance the efficiency of APCs and alter the microenvironment of the liver. However, inflammatory cytokines such as IFNs and tumor necrosis factor-α (TNF-α) can induce immune tolerance[39-41]. Many immune cells and signaling pathways in the liver contribute to immune responses against HBV infection. These immune responses are contact-dependent and can be affected by environmental factors. Inhibitory molecules are produced by hepatic stellate cells, Kupffer cells, T-regulatory cells and myeloid-derived suppressor cells. Through a contact-dependent manner, NK cells can kill HBV-specific CD8$^+$ T-cells. Moreover, HBV-specific CD8$^+$ T-cells can be suppressed by inhibitory ligands such as programmed death-ligand 1 (Figure 1)[6,42]. However, considering HBV has adaptive and active strategies to evade innate immune responses and negative feedback exists in all immune responses, immune regulator therapy targeting only a single pathway is unlikely to be effective in treating HBV infection.

Host genetic variations associated with HBV infection

Researchers have made great efforts to confirm the associations between HBV infection and host immunogenetics. Host genetic variants, including mutations in TLRs, HLAs, vitamin D-related genes, cytokine and chemokine genes, microRNAs, and HBV receptor sodium taurocholate co-transporting polypeptide, have been observed to impact the outcomes of HBV infection (Table 2)[43-46]. The HLA genes are divided into two classes: HLA-class I (HLA-A, B, C, E, F and G) and HLA-class II (HLA-DP, DQ, DR, DM and DO). Polymorphisms in HLA genes were shown to be significantly associated with the

Table 2 Host genetic variants associated with hepatitis B virus infection[43-46]

Gene ontology	Gene	Genetic determinants (SNP/Hap/CNVs)
HLA	*HLA-A*	*33:03:01
	HLA-B	*13:01:01
	HLA-B	*07, *58
	HLA-C	Leu-15, rs2853953, rs3130542
	HLA-DOA	rs378352
	HLA-DP	rs9366816
	HLA-DPA1	rs3077, rs2301220, rs2395309, rs9277341
	HLA-DPB1	G-A-G-A-T-T[1], G-G-G-G-T-C[2], rs2281388, rs9277535, rs9277542, rs9277534, *0201, *0401, *0901, positions 84–87, *0402, *0501, *0201-*0401, *0201-*0402, *0201-*0501, *0401-*0402, *0401-*0501, *0402-*0402, *0402-*0501, *0501-*0501, *0501- *0901, *0501*others, rs9277378, rs10484569, rs3117222, rs9380343, rs3135021
	HLA-DQ	rs9275319, rs9275572, A1*0101-B1*0501, A1*0102-B1*0303, A1*0102-B1*0604, A1*0301-B1*0601, A1*0102-B1*0602, A1*0102-B1*0602, A1*0301-B1*0302, A1*0301-B1*0303, A1*0301-B1*0401, A1*0501-B1*0301
	HLA-DPA1/ DPB1	A-A[3], A-A[4], T-A-T[5],C-A-T[6], A-A-C-T[7], A-A-C-C//A-G-T-G-C-C[8], A-A-C-T//A-G-T-G-C-C[9], G-G-T-C//A-G-T-G-C-C[10], A1*0103-B1*0401, A1*0103-B1*0402, A1*0202-B1*0301, A1*0202-B1*0501
	HLA-DQA2	rs9276370
	HLA-DQB1	*0201, *0301, *0303, *0502, *0604, rs2856718, *0401-*0501, *0402-*0402, *0402-*0501, *0501-*0501
	HLA-DQB2	rs7453920, rs7756516
	HLA-DRB1	*13
	HLA-DP/DQ	T-T-G-A-T[11], T-T-G-G-T[12], carrying 4–6 variant alleles, G-A[13], A-G[14], A-A[15]
	HLA-J	rs400488
Cytokines	*IL-10*	−592
	IL-10RB	rs2834167
	IL-12B	rs3212227
	IL-16	rs11556218
	IL-21	rs2221903
	IL-12B	rs3212227
	IL-18	−137
	IFN-γ	+874
	IFN-α2	p.Ala120Thr
	IFN-αR2	rs1051393, rs12233338
	IFNLR1	rs4649203, rs7525481
	IFN-γR1	rs3799488
	IFN-γR2	rs1059293
	TNF-α	T-C-C-G-G-G[16], C-A-C-G-G-G[17] ,−238, −308, −857, −863
	TGF-α	+106151, +103461, A-T-G-T-T-T-T-C-T[18]
Chemokines	*CCR5*	Δ32
TLRs	*TLR-3*	rs3775291, rs1879026
	TLR-9	rs352140
MicroRNAs	*miR-30a*	rs1358379
	miR-101-2	T-C[19], rs12375841
	miR-106b-25	rs999885

	miR-122	rs3783553, rs4309483
	miR-196a-2	rs11614913
	miR-let-7c	rs6147150
	miR-219-1	rs107822, rs213210, rs421446, C-A-C[20], T-G-T[21]
	miR-323b	rs56103835
	miR-423	rs6505162
	miR-492	rs2289030
Others	*C2*	p.Glu318Asp, rs7746553, rs9267673, rs9267665, rs9267677, rs9279450, rs10947223
	CD40	rs1883832
	CFB	rs12614
	CTLA-4	C-A-C-C-G[22], T/C-A-C-C-G[23], T-A-C-C-A[24], rs231775,rs3087243, rs5742909
	EHMT2	rs7887, rs652888, rs35875104, rs41267090
	ESR1	+29
	IFN4/HLA-DQ	rs12971396-rs9275319, rs12971396-rs12979860-rs9275319
	INST10	rs7000921
	KIF1B	A-T-A[25]
	MCP1	−2518, −2518/−2076(−/ht2), −2518/−2076(ht2/ht2)
	MIF	rs755622
	MxA	−123
	MX1	rs467960
	NF-κB	rs2233406, rs3138053
	NLRX1	p.Arg707Cys
	NOTCH4	rs422951
	NTCP	rs2296651, rs943277, rs4646285
	OCT4	rs13409, rs885952, rs879882, rs1265163, rs2394882, rs3094193, rs3130501, rs3130503, rs3130931, rs3132526, rs3757349, rs9263800, rs117265349
	PAPL	rs423058
	SOCS3	C-C[26], T-T-C-T-A[27], rs111033850, rs12953258,
	SPP1	−1800
	STAT4	C-T-C-T-T[28], rs7574865,rs8179673, rs10168266, rs11889341,
	TCF19	rs1419881
	TMEM2	p.Ser1254Asn
	TMEM2/IFNA2/ NLRX1/C2	p.Ser1254-Asn/p.Ala120Thr/p.Arg707Cys/p.Glu318Asp
	UBE2L3	rs2266959, rs4821116
	VARS2	rs1043483, rs1264295, rs2249464, rs2517459, rs2532932, rs9394021,
	VDR	FokI
	ZNRD1	G-G-A[29], rs3757328

Haplotypes:
[1]G-A-G-A-T-T, rs9277535-rs10484569-rs3128917-rs2281388-rs3117222-rs9380343.
[2]G-G-G-G-T-C, rs9277535-rs10484569-rs3128917-rs2281388-rs3117222-rs9380343.
[3]A-A, rs3077-rs9277535.
[4]A-A, rs2395309-rs9277535.
[5]T-A-T, rs3077-rs9277378-rs3128917.
[6]C-A-T, rs3077-rs9277378-rs3128917.
[7]A-A-C-T, rs2395309-rs3077- rs2301220-rs9277341.

[8]A-A-C-C//A-G-T-G-C-C, rs2395309-rs3077-rs2301220-rs9277341//rs9277535-rs10484569-rs3128917 -rs2281388-rs3117222-rs9380343.
[9]A-A-C-T//A-G-T-G-C-C, rs2395309-rs3077-rs2301220-rs9277341//rs9277535-rs10484569-rs3128917 -rs2281388-rs3117222-rs9380343.
[10]G-G-T-C//A-G-T-G-C-C, rs2395309-rs3077-rs2301220-rs9277341//rs9277535-rs10484569-rs3128917 -rs2281388-rs3117222-rs9380343;
[11]T-T-G-A-T, rs9276370-rs7756516-rs7453920- rs9277535-rs9366816.
[12]T-T-G-G-T, rs9276370-rs7756516-rs7453920-rs9277535-rs9366816.
[13]G-A, rs2856718- rs9275572.
[14]A-G, rs2856718- rs9275572.
[15]A-A, rs2856718- rs9275572.
[16]T-C-C-G-G-G, -1031/-863/-857/-308/-238/-163.
[17]C-A-C-G-G-G, -1031/-863/-857/-308/-238/-163.
[18]A-T-G-T-T-T-T-C-T, +88344/+102906/+103432/+103437/+103461/+104261/+104802/+106151/+106318.
[19]T-C, rs12375841-rs17803780.
[20]C-A-C, rs421446-rs107822-rs213210.
[21]T-G-T, rs421446-rs107822-rs213210.
[22]C-A-C-C-G, -1722/-1661/-658/-319/+49.
[23]T/C-A-C-C-G, -1722/-1661/-658/-319/+49.
[24]T-A-C-C-A, -1722/-1661/-658/-319/+49.
[25]A-T-A, rs17401966-rs12734551-rs3748578.
[26]C-C, rs111033850-rs12953258.
[27]T-T-C-T-A, -1800/-1627/+4645/+5806/+6139.
[28]C-T-C-T-T, rs8179673-rs7574865-rs4274624-rs11889341-rs10168266.
[29]G-G-A, rs3757328-rs6940552-rs9261204.
SNPs/Hap/CNVs: Single nucleotide polymorphisms/Haplotype/Copy number variations; HLA: Human leukocyte antigen; TLR: Toll-like receptors.

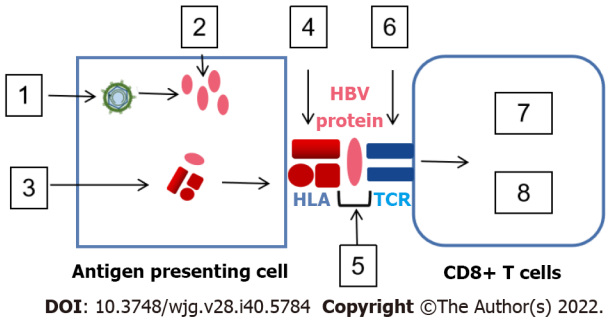

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Figure 2 Mechanism of immune escape in antigen-presenting cell/hepatitis B virus special T-cell. Hepatitis B virus (HBV)-infected hepatocytes produce various HBV antigens that are swallowed and digested by antigen-presenting cells (APCs), producing HBV peptide/human leukocyte antigen (HLA) complexes. Antigen processing escape mutants, down-regulating HLA expression and mutation of HLA binding residues may appear in APCs. The HBV peptide/HLA complexes are transferred to the surface of APC and make contact with T-cell receptor (TCR) on the surface of $CD8^+$ T-cells. Masking HLA/TCT binding residues with N-linked glycosylation and mutation of TCR binding residues influences TCR affinity/avidity, leading to $CD8^+$ T-cell stimulation or inhibition. The square icon displays: (1) Increase in viral antigen; (2) Antigen processing escape mutants; (3) Down-regulation of HLA expression; (4) Mutation of HLA binding residues; (5) Masking of HLA/TCT binging residues with N-linked glycosylation; (6) Mutation of TCR binding residues; (7) Stimulation induced by cytokine production and cytolytic activity; and (8) Inhibition caused by exhaustion, anergy and tolerance. HBV: Hepatitis B virus; TCR: T-cell receptor; HLA: Human leukocyte antigen.

pathogenesis of HBV infection[47,48]. Studies have also reported that the genes encoding cytokines such as interleukins, TNF-α, IFNs and TGF-β, can influence the immune state of CHB patients[49-53]. In a study by Nitschke *et al*[54], the authors confirmed that specific HLA class I alleles restricted the efficacy of HBV-specific $CD8^+$ T-cells[54], indicating that the host's immune-related genes can affect the outcome of hepatitis B infection.

Researchers have observed significant differences in HBV infection rates between Western and East/Southeast Asian populations. Prior to HBV vaccination programs, the prevalence of HBsAg was less than 1% in the Caucasian population but higher than 10% in the Chinese population[43]. This difference was investigated in several studies, which showed that HLA molecules in European, Caucasian, Middle East, African-American and Asian populations affected the rate of HBV infection[43,55,56]. In addition, discordances in HBV-specific $CD8^+$ T-cell repertoires observed in different races, i.e., between Chinese and Caucasian populations, could be related to race-dependent HLA gene variants, leading to the different T-cell responses observed between different populations and ethnicities[57].

Thus, these findings underline the complexity of HBV-specific immune control efficacy, which is influenced by HBV components, negative feedback regulation in immunity and host genetic variants (Figure 3).

Heterogeneity of immune control in HBV infection

Heim *et al*[6] reviewed the heterogeneity of HBV-specific $CD8^+$ T-cells and described the functional

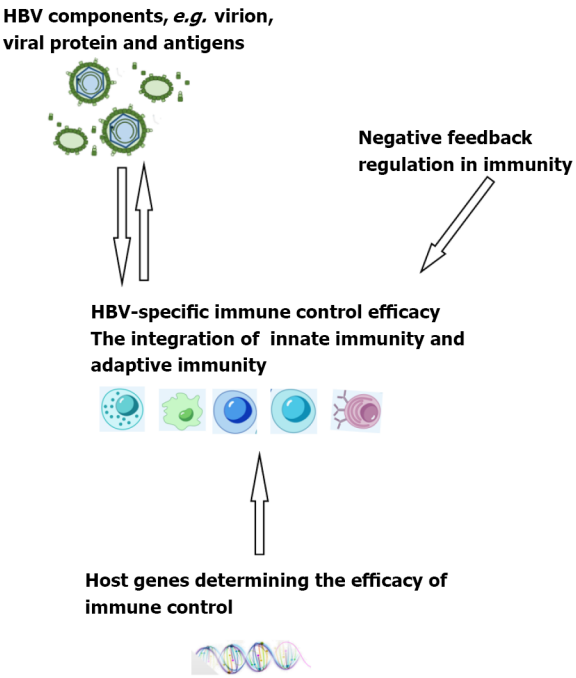

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Figure 3 Interplay between hepatitis B virus-specific immune control, hepatitis B virus components, and negative feedback regulation in immunity and host genes. HBV: Hepatitis B virus.

deficiencies and distinct phenotypical characteristics associated with HBV-specific $CD8^+$ T-cells exhaustion. T-cell impairment demonstrates hierarchical and progressive loss in antiviral functions, from functional suppression to physical deletion in T-cells, which depend on the quantity of HBV antigens and the duration of T cell exposure to these antigens[31,58]. Moreover, exhausted $CD8^+$ T-cells do not represent a homogeneous T-cell population but are rather heterogeneous in function and phenotype. Kuipery *et al*[5] proposed concepts related to the heterogeneity of immune responses in CHB patients[5]. Based on reports from literature, we summarized the following concepts to improve our understanding on the heterogeneity of immune control.

Figure 1 illustrates the activation and inhibition of immune signaling pathways that overlap in HBV infection, multiple immune cell populations and integrated signals pathways that simultaneously respond to the stimulation of HBV components, and the ability of HBV-specific immune control depends on the overall immune responses of an individual rather than the capability of single immune cells or a single immune pathway.

Based on the immune escape mechanisms of APCs/$CD8^+$T-cells presented in Figure 2, the heterogeneous immune responses of T-cells are related to very fine mechanisms of immune regulation that influence the immune control of HBV-specific T-cells, or T-cells sensitivity/inhibition.

Figure 3 outlines the reasons for heterogeneous immune control. The overall immune control against HBV in an individual is influenced by HBV, negative feedback regulation in the immune signaling network and host genetic variants. Therefore, any changes in these three countenances could lead to fluctuations affecting the effectiveness of immune control. Thus, CHB infection is divided into four clinical phases: Immune tolerant with HBeAg-positive, HBeAg-positive immune-activation, inactive carrier with HBeAg-negative, and HBeAg-negative immune-activation. Further, heterogeneous immune controls can lead to different clinic phases and disease outcomes such as acute hepatitis, chronic hepatitis, cirrhosis, hepatocellular carcinoma and hepatic failure[43].

Reports have shown that host genetic polymorphisms are crucial determinants influencing HBV infectious rate and disease outcomes in Caucasian, Saudi Arabian, African-American, European, and Asian populations[56,59,60]. In addition, the host genetic variants of individuals from the same race can influence disease outcomes and the efficacies of antiviral therapy.

Researcher and immunologists who often overlook immune compartmentalization should be cautious when interpreting findings from the peripheral blood of patients.

IFN-α TREATMENT FOR CHB

Definition of partial cure and functional cure

A complete cure from HBV infection is currently hypothesized to be possible if the cccDNA and integrated HBV DNA are eliminated from hepatocytes. However, this is challenging as cccDNA can still persist in patients despite spontaneous recovery from an acute HBV infection. Since 2017, some advances in CHB therapeutics have been achieved[61], and researchers have proposed new definitions of cure. For instance, a partial cure has been defined as persistently undetectable HBV DNA and HBeAg in patients' serum after the completion of a limited course of antiviral therapy. Functional cure has been defined as sustained and undetectable HBV DNA and HBsAg in patients' serum, with or without seroconversion to hepatitis B surface antibody (anti-HBs), after a limited course of therapy. Complete sterilizing cure has been defined by the absence of HBsAg and eradication of HBV DNA, including HBV virion, integrated HBV DNA and cccDNA in the liver and serum of patients. However, because current treatments cannot deliver complete sterilizing cure, functional cure is the selected goal of therapy.

NAs and immunotherapy

Therapeutic strategies for CHB can be classified into two categories: NAs targeting HBV replication and immune modulators targeting immune control. NAs can cause rapid decay of HBV DNA in the peripheral blood but cannot completely eradicate HBsAg from patients' serum and cccDNA in hepatocytes[62,63]. Even though the virus might be undetectable in the serum after long-term treatment with NAs, low-level viral replication still persists due to the conservation of cccDNA in the nucleus of hepatocytes. Thus, prolonged NAs treatments are rarely associated with CHB cure and inevitably result in either NAs resistance or viral relapse[62-66].

In contrast, immunotherapy can target the innate and adaptive immunity to reinvigorate the host immune response to long-term inhibition of viral replication. Therapeutic strategies involving innate immunity include activating pattern recognition receptors (*i.e.*, TLRs and RIG-I), enhancing cytokine secretion, and improving the efficacy of NK cells. For adaptive immunity, therapies are directed at restoring the effects of HBV-specific T-cells and B-cells. Clinically, although immunotherapy can lead to sustained HBsAg loss, it cannot eradicate cccDNA from hepatocytes. Thus, being the more promising treatment compared to NAs, the ultimate goal of immunotherapy is currently targeted at achieving functional cure, enabling spontaneous control of HBV replication and maintaining disease remission without antiviral therapy[3]. Hoogeveen and Boonstra[32] reported that immunomodulators regulating a single immune pathway might not restore antiviral immunity as multiple immune pathways in the host enhance specific HBV immune responses[32].

Mechanism and clinical application of IFN-α therapy

IFNs are produced and released by immune cells in response to HBV components[67,68]. Of the known IFNs, IFN-α has broad-spectrum effects on viruses and tumors, and contributes to immune regulation by suppressing viral replication and cell growth. The antiviral function of IFN-α is cascaded by binding to IFN receptors on immune cells, activating signal transcription pathways, and inducing ISGs expression and related product secretion. Various ISGs products were found to inhibit different stages of the viral life cycle[69]. Further, epigenetics was shown to play a critical role in regulating cccDNA transcription[70-73]. IFN-α can regulate the epigenetic repression of cccDNA transcription by inducing cccDNA-bound histone hypo-acetylation and increasing the recruitment of transcription co-repressor on cccDNA[63,65,73]. In addition, IFN-α can upregulate HLAs expression to activate innate and adaptive immune responses. Many researchers have investigated the host genes associated with IFN-α treatment outcomes and observed its genetic polymorphisms[43,44,74-78] (Table 3).

As described earlier, due to negative feedback mechanisms, immune modulators targeting a single signal pathway might not improve the overall and long-term immune responses[61]. In this regard, the advantages of IFN-α treatment are that IFN-α can simultaneously affect multiple immune pathways and various immune cell populations in the host and integrate signals to improve the efficacy of immune control[10,11,15,36]. Currently, only IFN-α treatment was found to improve the efficacy of immune control[64,65].

IFN-α has been approved for the treatment of hepatitis B for over 20 years[36,61,65]. Compared with NAs, IFN-α has shown better efficacy in HBeAg seroconversion and HBsAg loss, with no risk of drug resistance[79]. For groups of patients with good prognoses, such as Caucasians, young age, low viral load, and females, IFN-α treatment has been more effective[80,81]. One study reported functional cure in 10%–20% of Caucasians who underwent IFN-α treatment, while it was < 5% in Asian patients[82]. However, most of the underlying mechanisms of IFN-α therapy are still unclear. More research is needed to investigate these significant differences in IFN-α efficacy between different groups of patients.

Table 3 Host genetic variants associated with interferon-α therapy[43,44,74-78,91]

Gene ontology	Gene	Genetic determinants (SNP/Hap/CNVs)
HLA	*HLA-DP*	rs3077, rs9277535
	HLA-DQA1-DQB1-DRB1	*0302-*0303-*09
	HLA-DQB1	*0303
	HLA-DRB1	*08
	HLA-A, B, C	*1101-*4601-*0102
Cytokines	*IL-28B*	A-C[1],rs8099917, rs12979860, rs12980275
Others	*CYP24A1*	rs2248359
	CYP27B1	rs10877012, rs4646536
	G3BP2	rs3821977
	OAS	G-T-G-A[2], C-C-T-A[3], C-C-C-A[4], A-C-T-A[5]
	OAS3	rs2072136
	PRELID2	rs371991
	STAT4	rs7574865
	TRAPPC9	rs78900671
	VDBP	rs7041
	VDR	rs1544410, rs731236, rs11568820, rs10735810

Haplotypes:
[1]A-C, rs12980275-rs12979860.
[2]G-T-G-A, rs3177979-rs1293747-rs4767043-rs10849829.
[3]C-C-T-A, rs2285934-rs2072138-rs2072136-rs10849829.
[4]C-C-C-A, rs2285934- rs2072138-rs2072136-rs10849829.
[5]A-C-T-A, rs2285934-rs2072138-rs2072136-rs10849829.
SNPs/Hap/CNVs: Single nucleotide polymorphisms/Haplotype/Copy number variations; HLA: Human leukocyte antigen.

IMPROVEMENT OF HBV-SPECIFIC IMMUNE CONTROL BY IFN-α RETREATMENT

IFN-α retreatment

In 1996, although IFN-α was already being used to treat CHB patients, only 20%-30% of the patients achieved viral suppression or partial cure with single IFN-α treatment. Then, it was found that IFN-α retreatment in these remaining patients could enhance treatment outcomes. Thus, multiple courses of IFN-α were implemented to treat these patients and researchers observed that three courses of IFN-α treatment were effective in treating HBeAg positive or negative patients. With multiple frequencies of IFN-α treatment, partial or functional cure rates gradually increased to approximately 25%-40% but were mostly observed in patients of white race[10-14] (Table 4).

By 2008, failures from combination therapy with nucleoside and nucleotide were reported, and multi-drug resistance with NAs treatment started to increase in China. Comparatively, IFN-α retreatment was associated with safe stopping of NAs administration and induced better-sustained responses to IFN-α [15]. Thus, when researchers started to investigate the effects of increasing the frequency and extending the total course of IFN-α treatment and found that these could significantly improve the rate of functional cure in Asian and Caucasian patients[73-75,80-83]. IFN-α retreatment was recommended by the Asian-Pacific clinical practice guidelines[16-18] and by Yin *et al*[64] (Table 4). Based on these promising findings and recommendations, we estimate that more CHB patients in Asia would receive IFN-α retreatment and experience better antiviral efficacy.

Interplay between IFN-α retreatment and the HBV-specific immune control

Recently, more and more HBV-specific immune mechanisms have been discovered, inspiring clinicians and immunologists to collaborate to study the interplay between immune mechanisms and clinical events in hepatitis B. Chang *et al*[84] summarized the relationship between hepatitis flare and immune responses in CHB patients to explore the underlying immune mechanism of hepatitis flares[84]. The observations made from the strategy of Chang's *et al*[84] were used below to discuss the immune control involved in IFN-α retreatment.

Table 4 Summary and timeline of interferon-α retreatment for chronic hepatitis B virus infection

Ref.	Year	The regimens of INF-α retreatment
van Zonneveld *et al*[10]	2004	One hundred and sixty-five patients received their first IFN-α treatment. Of them, 48 non-responders received 2-3 courses of IFN-α
Manesis and Hadziyannis[11]	2001	Two hundred and sixteen patients received IFN-α therapy, of whom 51 received a second IFN-α treatment and 9 were treated with three courses of IFN-α
Niederau *et al* [12]	1996	One hundred and three cases were treated IFN-α. Of them, 29 received a second course of therapy, and 17 received a third course of IFN-α
Carreño *et al*[13]	1999	Fifty-seven patients underwent a course of IFN-α therapy. Of them, 27 received a second course of IFN-α; and 30 others who did not receive IFN-α retreatment served as controls. IFN-α retreatment was found to enhance the antiviral effects against HBV
Ballauff *et al*[14]	1998	After all the children received the first course of IFN-α treatment, 15 were given the second course of IFN-α (study group), while the other 19 did not receive the second course of IFN-α, serving as the control group. HBV DNA clearance and anti-HBe seroconversion occurred in 5 of 15 children (33%) in the study group and 5 of 19 children (26%) in the control group
Yin and Zhong [15]	2016	Inappropriate therapies induced HBV resistance, multi-drug resistance and failure of combination therapy with nucleoside plus nucleotide in China. An IFN-α regimen, *i.e.* (1) Switch from nucleoside plus nucleotide to IFN-α therapy; and (2) subsequent IFN-α retreatment, was used to treat these patients. IFN-α switching therapy leaded to safe cessation of nucleoside plus nucleotide combination therapy, and IFN-α retreatment induced sustained response of IFN-α
Sarin *et al*[16]	2016	Asian-Pacific clinical practice guidelines 2015 recommended IFN-α retreatment to treat chronic HBV infection
Liaw *et al*[17]	2012	Asian-Pacific clinical practice guidelines 2012 recommended IFN-α retreatment to treat chronic HBV infection
Liaw *et al*[18]	2008	Asian-Pacific clinical practice guidelines 2008 recommended IFN-α retreatment to treat chronic HBV infection
Yin *et al*[64]	2021	IFN-α retreatment was recommended for patients on entecavir or tenofovir monotherapy with persistent low-level viremia

IFN-α: Interferon-α; HBV: Hepatitis B virus.

Asymptomatic persistence of cccDNA in the liver of patients who had acute hepatitis B or self-limiting HBV infection, despite the resolution of HBV infection[3], indicates that the host's immune system can fully inhibit HBV replication and that the specific HBV immune control in these individuals had an overwhelming advantage over HBV replication. In addition, investigations on the heterogeneous immune control of HBV infection have shown that the heterogeneity depends on the interplay between the host, virus and therapy, including host genes and immunity state, HBV load, duration of HBV infection, and IFN-α treatment and course of treatment[5,6,31,58]. However, the exact mechanism *via* which IFN-α retreatment exerts its benefits is yet to be fully elucidated.

Apart from HBV load, HBsAg and HBeAg, markers associated with the immune control of IFN-α treatment are lacking. In a study by Konerman and Lok[79], the authors proposed a scoring system that could help assess the efficacy of immune control[79]. The type of immune control could be estimated from a patient's HBV DNA, HBeAg seroconversion, HBsAg loss and times of IFN-α treatment, based on the following criteria: (1) Patients with acute hepatitis B or self-limiting HBV infection could achieve automatic cure without IFN-α treatment; (2) Patients with acute hepatitis B could achieve functional cure with one time IFN-α treatment; (3) CHB patients with one time IFN-α treatment could achieve functional cure; (4) CHB patients with one time IFN-α treatment could achieve partial cure; (5) CHB patients with multiple times of IFN-α treatment could achieve functional cure; and (6) CHB patients with multiple times of IFN-α treatment could achieve partial cure. These criteria suggest a step-like decline in immune control with increasing infection severity.

Clinical studies have confirmed that IFN-α retreatment could gradually increase the rate of partial and functional cure (Table 4)[10,11,13,15,36] and that CHB patients often have diverse HBV-specific immune control. Since 2016, researchers in China have reported numerous findings from clinical trials in which patients with inactive HBsAg carriers or low-level viremia were selectively enrolled and treated with IFN-α to evaluate their functional cure rate[85-88]. However, it should be noted that these were performed under trial settings because clinical guidelines do not recommend IFN-α therapy for the treatment of CHB in these patients[16,89,90]. A high rate of functional cure, 44.7%-84.2% of HBsAg loss and 20.2%-68.2% of HBsAg seroconversion were reported, in which a distinctive pattern of immune control whereby a close correlation between lower HBsAg at baseline and higher rates of HBsAg loss or HBsAg seroconversion was observed[85-88]. This pattern also appeared in the final course of IFN-α retreatment in previous studies[10,11,13,15,36], indicating that reducing HBsAg could be a prerequisite for achieving functional cure during IFN-α retreatment.

Taken together, current literature indicates that IFN-α retreatment could gradually enhance the overall immune control and improve the antiviral efficacy of IFN-α in CHB patients (Figure 4).

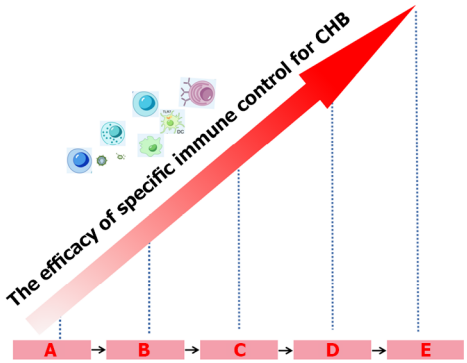

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Figure 4 Interferon-α retreatment improves the efficacy of hepatitis B virus-specific immune control. Interferon-α (IFN-α) retreatment can lead to various treatment outcomes, namely no response, hepatitis B virus (HBV) decline, partial cure and functional cure. Multiple frequencies of IFN-α treatment can potentially restore specific immune control to HBV infection and simultaneously increase the rate of partial cure and functional cure. A: Exhausted immune control to HBV before IFN-α therapy (Baseline); B: Without HBV decline following IFN-α therapy; C: HBV decline; D: Partial cure; E: Functional cure. CHB: Chronic hepatitis B.

CONCLUSION

The interactions between immune cells and cytokines form a complex immune response network that exerts immune control over HBV infection. The efficacy of HBV-specific immune control is affected by HBV components, negative feedback regulation in the immune system, host genetic variants, and heterogeneity in the function and phenotype of immune control to HBV. Treatment with IFN-α can simultaneously affect multiple immune pathways and various immune cell populations in the host and integrate signals to improve the efficacy of immune control. Clinically, increasing the frequency and extending the total course of IFN-α retreatment have improved functional cure rates, indicating that IFN-α retreatment could gradually enhance the overall immune control. Further research on IFN-α retreatment could help promote this strategy in CHB patients with.

Altogether, this article outlined immune control without detailed discussions on immunity-related markers during immune transformation. In future studies, the discovery of detailed markers associated with immune transformation could provide important clues in understanding the underlying mechanism of immune control to improve the treatment of HBV infection.

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FOOTNOTES

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Abstract

The whole world has been continuously afflicted by the coronavirus disease 2019 (COVID-19) pandemic for the past 3 years. Many countries have tried many methods to control this virus infection with varying successes and failures. The gut microbiota is a biosystem spanning the entire length of the digestive tract and playing important roles in health and disease. It is much affected by COVID-19. In return it also substantially impacts infection. In particular, the gut microbiota has established a bidirectional interaction with the COVID-19 vaccines, enhancing or reducing vaccine efficacy by virtue of its varying components. Conversely, COVID-19 vaccines also make a substantial impact on the gut microbiota, reducing its overall population and biodiversity. It is hoped that by exploring and harnessing this bidirectional interaction we may break new ground and develop new methods to prevent and treat this formidable virus infection.

Key Words: Gut microbiota; COVID-19 vaccines; Vaccine interactions; Gut microbiota alterations

Core Tip: The gut microbiota is a biosystem spanning the entire length of the digestive tract and playing important roles in health and disease. It is much affected by coronavirus disease 2019 (COVID-19). In return it has impacts on the infection and a bidirectional interaction with the COVID-19 vaccines has been established, which enhances or reduces vaccine efficacy. Conversely, COVID-19 vaccines also make a substantial impact on the gut microbiota, reducing its overall population and biodiversity. By exploring and harnessing this bidirectional interaction we may hopefully break new ground and develop new methods to fight this formidable pandemic.

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INTRODUCTION

The gut microbiota is a highly important and intriguing biosystem extending throughout the alimentary tract, covering an area of 400 square meters and with a biodiversity spanning over 2000 species, including protozoa, fungi, bacteria and viruses[1]. These organisms could be intraluminal or attached to the linings of the gut. Some even occupy an intracellular or subepithelial intercellular residence, while others enter the tissue fluid, the lymphatics and even the blood stream[1].

With such a background, the gut microbiota is constantly engaged in interactions with the various systems of the host and plays an important part both in health and in disease. In health, it is an integral part of digestion, absorption and nutrition. The contribution of gut microbiota in the production of vitamins of the B family and vitamin K has become common knowledge. More recently, it has been shown that gut microbiota, with their rich endowment of enzymes, could digest far more varieties of carbohydrates than their host could do alone[2]. Gut microbiota could also synthesize essential amino acids from inorganic nitrogen so that the host could survive even on a protein-free diet.

In fat metabolism, some bacteria are able to synthesize long chain fatty acids, contributing to the host energy supply as well as obesity, while many others, typically the strict anaerobes, ferment carbohydrates into short chain fatty acids (SCFAs). SCFAs inhibit histone deacetylase and activate G-protein coupled receptors with benefits in antioxidant, anti-inflammatory, antitumorigenic and antide-generative functions[3]. Indeed, research has shown the potential of fecal SCFA content as a marker of intestinal health, being at a lower level in colon cancer *vs* healthy controls[4]. Further studies of serum free fatty acid profiles especially the SCFA profiles show distinct patterns for healthy colon, colonic adenomatous polyps, colon cancer and coeliac disease reflecting the differences of gut microbiota among these conditions[5].

It is beyond the scope of this review to cover all aspects of the gut microbiota in health and disease but rather to focus on the intriguing interaction between the gut microbiota and coronavirus disease 2019 (COVID-19). The COVID-19 pandemic has been continuously ravaging the whole world for the past 2.5 years. Up to August 22, 2022, the World Health Organization's statistics showed the cumulative number of infected cases exceeded 211 million and was still increasing at 4.5 million *per* week, while the cumulative number of deaths had exceeded 4.4 million and was still increasing at around 68000 *per* week[6]. Various well-established methods of infection control have been tried, mostly with only partial success, and complete control remained elusive. Meanwhile, the phenomenon of "pandemic fatigue" has set in, and people become less inclined to adhere to control measures instead of tightening infection control measures[7]. In this review, aspects of gut microbiota that are relevant to the pandemic and its potential contribution to pandemic control was the focus.

GUT MANIFESTATION IN COVID-19 AND MICROBIOTA ALTERATIONS

The earliest report of COVID-19 in The Lancet on January 24, 2020 on the first 41 cases in Wuhan stated that diarrhea was the presenting symptom in only 1 case[8]. Three months later, when a New York center reported its first 393 cases of COVID-19[9], diarrhea was the presenting symptom in 23.7%. The incidence of diarrhea in the two districts remained almost unchanged over the next year with China at 3.80% diarrhea among 1141 cases[10] and New York at 20.14% diarrhea among 278 cases[11]. The difference is obvious and significant. Conceivably the diet in the Wuhan population is quite different from that in New York. It is probable that the gut microbiota in these two localities would also have a considerable difference, offering a plausible explanation of the increased diarrhea among the New York patients. In addition a Western diet, rich in processed meat but deficient in microbiota accessible carbohydrates, would have lower biodiversity[12] and less favorable to health. Unfortunately for the research investigator, most of these early studies did not report details of gut microbiota status, and the opportunity to study the influence of the gut microbiota on the early phase of the pandemic was lost.

GUT MICROBIOTA ALTERATIONS AND COVID-19 SEVERITY

By 2021, it became obvious that gut involvement and diarrhea were associated with greater severity of COVID-19[13]. An investigation in Hong Kong further demonstrated that certain components of the gut microbiota with immune-modulatory potential were depleted in severe and long-lasting COVID-19, notably *Faecalibacterium prausnitzii*, *Eubacterium rectale* and bifidobacterial species[14]. The investigators proposed that depletion of these bacteria could be taken as biomarkers predictive of severe and prolonged COVID-19. It is tempting to suggest that further studies might even explore the therapeutic value of replenishing these organisms to mitigate the disease.

GUT MICROBIOTA AND THE IMMUNE SYSTEM AND IMMUNE THERAPY

The interaction between the gut microbiota and the immune system goes far beyond the three groups of bacteria mentioned in the last section. In fact, beginning with the first colonization of the gut at birth or even before birth, the gut microbiota continuously evolve and influence the development of the host immune system, fostering reactivity against pathogenic invaders and tolerance towards harmless colonizers or beneficial symbionts[15]. Such actions are mediated by both regulatory cytokines (like interleukin-10 and interferon-beta) and T regulatory cells. This not only promotes diversity and increases beneficial microbes but actually helps to reduce host autoimmune disorders.

On the other hand, antibiotics have been shown to reduce the gut microbiota in both quantity and diversity, with reduced efficacy of immune checkpoint inhibitors in immunotherapy of cancer, and fecal transplantation has been shown to successfully restore the immune therapeutic response in such patients[16].

GUT MICROBIOTA'S IMPACT ON COVID-19 VACCINES: EFFICACY AND SIDE EFFECTS

With the foregoing background we now come to the important consideration of controlling the ongoing COVID-19 pandemic. It is common knowledge that pandemic control rests on five pathways: (1) Isolation of patients at the infectious stage by quarantines and social distancing; (2) Blocking the routes of infection by masking, air filtering/exchanging and sanitation; (3) Building up resistance among the population with vaccination; (4) Development of effective medicines to cure the infected patients and eliminate the carrier status; and (5) Letting the pandemic run its natural course, eliminating all susceptible components of the population and leaving those with inborn or naturally acquired immunity to survive.

The first four measures at present run into many obstacles, including social, economic, political, even personal egocentric considerations and biased sentiments. What is left is the fifth choice, otherwise called "herd immunity," a rather primitive, counter-intuitive and inhuman approach. Fortunately, amidst these dark looming clouds appears a silver lining. The gut microbiota might not only modify the COVID-19 disease but actually improve the efficacy and reduce the side effects of its vaccines, winning more skeptics to accept this highly important preventive measure.

This seminal work, a combined effort of the two universities in Hong Kong, was reported by Ng *et al*[17] and published on February 9, 2022[17]. It showed that for vaccine recipients of the inactivated virus, CoronaVac, the relatively low induction of neutralizing antibodies could be increased with a higher level of *Bifidobacterium adolescentis* (*B. adolescentis*) in the gut microbiota, while *Bacteroides vulgaris*, *Bacteroides thetaiotaomicron* and *Ruminococcus gnavus* were enriched in low responders. Another vaccine, the viral spike protein-encoded messenger RNA, BNT-162b, under the brand name Comirnaty, although capable of eliciting high antibody levels, could be further improved by the abundance of flagellate and fimbriate bacteria like *Roseburia faecis*. In addition, for both vaccines, enrichment of *Prevotella copri* and two *Megamonas* species led to fewer side effects, likely due to the anti-inflammatory influence of these organisms.

Interestingly, the role of *B. adolescentis* in CoronaVac seems very specific. So far, according to these researchers, any other species of the same *Bifidobacterium* genus tested would not work. As the species *B. adolescentis* is not present in commonly available health food or probiotic preparations, there seems no way to simply make an off-the-counter purchase of "health foods" to obtain such benefit. By contrast, the requirements of BNT-162b for *Roseburia faecis* seem less fastidious, and various bacteria with flagella and fimbriae might also impart benefit. Even *Bacteroides thetaiotaomicron*, known to be associated with low antibody production in CoronaVac, joins the company of antibody-enhancers for BNT-162b. This list may also include a minimal existence of *B. adolescentis* because the only BNT-162b recipient who failed to develop adequate antibody level was entirely devoid of *B. adolescentis*.

IMPACT OF COVID-19 VACCINES ON GUT MICROBIOTA: A BIDIRECTIONAL INTERACTION

Ng *et al*[17] not only showed the impact of gut microbiota on vaccine efficacy but also showed the impact of vaccination on gut microbiota 1 mo after delivering two doses[17]. For CoronaVac, only *Bacteroides caccae* was increased. For BNT-162b2, both *B. caccae* and *Alistipes shahii* increased. Common to both vaccines, a large number of species were diminished including *Adlercreutzia equolifaciens*, *Asaccharobacter celatus*, *Blautia obeum*, *Blautia wexlerae*, *Dorea formicigenerans*, *Dorea longicatena*, *Coprococcus comes*, *Streptococcus vestibularis*, *Collinsella aerofaciens* and *Ruminococcus obeum*[17]. There seemed to be a substantial loss of biodiversity, but no further elaboration on the clinical and pathological significance was mentioned. With such substantial changes in the gut microbiota one would expect some alterations in bowel habits after vaccination. On a theoretical basis, vaccine-induced loss of diversity would

Table 1 Diarrhea in persons aged 19-55 years after Pfizer-BioNTech coronavirus disease 2019 vaccination based on The Centers for Disease Control online published table[23] under the title "Vaccines and Immunizations"

Subjects	First injection number		Second injection number	
	Vaccine 2291	Placebo 2298	Vaccine 2098	Placebo 2103
Symptoms				
Any diarrhea	255 (11.10%)	270 (11.70%)	219 (10.40%)	177 (8.40%)
Severe diarrhea	3 (0.13%)	1 (0.04%)	4 (0.19%)	1 (0.05%)

increase the opportunity of pathogens to thrive in the intestine. There would be less competition for nutrition and for the niche of bacteria habitat, with less antagonistic factors produced by healthy bacteria such as bacteriocin and SCFAs to discourage the growth of pathogenic organisms[18]. With the proliferation of pathogenic organisms, the chance of diarrhea would be increased. Minor changes in bowel habits, however, tend to be under-reported, and severe diarrhea would tend to be so uncommon that it is often underpowered to establish a statistically significant conclusion. Table 1 was constructed from data published online by the Centers for Disease Control and Prevention (United States).

While there is no significant difference in mild diarrhea between recipients of vaccine and placebo, a signal of increased severe diarrhea among vaccine recipients seems to show up in the bottom row for both first and second injections, possibly reflecting the increase in severe diarrhea by 3.2 times (for first injection) to 3.8 times (for second injection) as a result of diminished diversity of gut microbiota. This table has two limitations. First, the actual number of severe diarrhea cases are too small for statistically significant computation. Second, no information is given for the composition of the microbiota of these patients, and it is not possible to relate the diarrhea to any particular organism or to the vaccine itself.

BIDIRECTIONAL INTERACTION BETWEEN GUT MICROBIOTA AND IMMUNE ACTIVITIES BEYOND COVID-19

Therapeutic agents, including vaccines, may have therapeutic value well beyond their originally intended effects. One of the best-known examples is the anti-tuberculosis BCG vaccine, whose role in protection against leprosy is well studied and documented[19]. For over 40 years it has also played a role in the treatment of non-muscle-invasive bladder cancer[20]. Indeed, many vaccines have been actively studied as a platform for anticancer treatment[21]. In certain areas, like hepatocellular carcinoma and carcinoma of the cervix, anti-viral vaccines have successfully prevented the cancer by preventing infection of the respective oncogenic virus. In other cancers, the mRNA technology has played a pivotal role in stimulating the patient's immune system to recognize and react against tumor-associated neo-antigens[22].

For three decades scientists have been struggling to iron out technical obstacles and the overall reluctance of recruiting the body's immune cells to produce an antigen and provoke an immune reaction, which is like retracing the steps of autoimmune disorders (the same sentiment still prevails among COVID-19 vaccine doubters today). Consequently, the predominant form of cancer immunotherapy at present is immune checkpoint inhibition, which involves abolishing major mechanisms of evasion of cancer from the host immunity *via* immune checkpoints. Even in this context, certain components of the gut microbiota, notably *Bifididobacterium longum* and *Akkermansia muciniphila* are found to be associated with good response to checkpoint inhibitors in melanoma and lung cancer, respectively, while lowered diversity and population of the gut microbiota under antibiotic treatment would have the opposite effect[16]. When the COVID-19 pandemic broke out, the mRNA-based vaccines, backed by years of research in previous anticancer immunotherapy, had a chance to be tested extensively and speedily with resounding success.

CONCLUSION

As mentioned earlier in the study of Ng *et al*[17], certain gut microbes could enhance vaccine efficacy in antibody stimulation while other microbes have the opposite effect[17]. Conversely, COVID-19 vaccines could impact the gut microbiota, enhancing the growth of some microbes but suppressing the growth of many others[17]. This bidirectional interaction between gut microbiota and COVID-19 vaccine is highly reminiscent of that between gut microbiota and anticancer immunotherapy. Conceivably, modifying the gut microbiota might enhance the vaccine-induced therapeutic value for both infection and cancer. It will take further study to understand and possibly harness such interactions and convert their potential therapeutic value into reality.

FOOTNOTES

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MINIREVIEWS

Improving the prognosis before and after liver transplantation: Is muscle a game changer?

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Abstract

Liver transplantation (LT) is currently the only curative treatment option for selected patients with end stage liver disease or hepatocellular carcinoma. Improving waiting list-mortality, post-transplant morbidity and mortality and refining the selection of the patients remain our current central objectives. In this field, different concepts dealing with nutrition and the muscle such as sarcopenia, malnutrition, frailty or myosteatosis have emerged as possible game changers. For more than a decade, many prospective studies have demonstrated that sarcopenia and frailty are major predictive factors of mortality in the waiting list but also after LT. Malnutrition is also a well-known risk factor for morbidity and mortality. Muscle composition is a newer concept giving insight on muscle quality which has also been shown to be linked to poorer outcomes. Each of these terms has a precise definition as well as pathophysiological mechanisms. The bi-directional liver-muscle axis makes sense in this situation. Defining the best, easy to use in clinical practice tools to assess muscle quality, quantity, and function in this specific population and developing quality prospective studies to identify interventional strategies that could improve these parameters as well as evaluate the effect on mortality are among the important challenges of today.

Key Words: Muscle; Liver; Transplantation; Sarcopenia; Myosteatosis; Frailty

Core Tip: Patients suffering from end stage liver disease currently have liver transplantation as the only curative treatment. In order to improve the pre and post transplantation management of patients, muscle related concepts such as sarcopenia, myosteatosis, frailty and malnutrition could be important potential game changers. Considering a bidirectional axis between the muscle and the liver, it is therefore justified to characterize the condition of the muscles adequately and to identify interventional strategies that could improve both muscle parameters and patient survival.

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INTRODUCTION

To date, despite medical developments in knowledge and drug treatments, liver transplantation (LT) remains the only curative treatment option for selected patients with acute liver failure, end-stage liver disease (ESLD), liver cancer and acquired or genetic metabolic liver disease[1]. ESLD is the most frequent indication for LT with alcohol-related liver disease as the main etiology[2]. Other common etiologies are metabolic dysfunction-associated fatty liver disease (MAFLD) and viral hepatitis[3,4]. Autoimmune related liver diseases and cholestatic diseases are rarer indications in Western countries. The epidemiology of LT has changed over the last 20 years since the selection of patients suffering from hepatocellular carcinoma (HCC) with the Milan criteria and the progressive eradication of hepatitis C virus since the universal use of direct-acting antivirals[5]. MAFLD is probably becoming one of the main indications for LT in parallel with the epidemic of obesity in Western countries. This disease can coexist with other causes and act as an important co-factor. This specific population represents a series of challenges in the pre-, peri- and post-transplant settings due to the presence of medical comorbidities that include obesity, type 2 diabetes and cardiovascular risk factors[6,7].

For years, graft allocation has been based on the model for end-stage liver disease (MELD) score that has been used to predict 3-mo mortality on the waiting list. The use of the MELD score has led to a better selection of patients requiring LT but also to a reduction in waiting time and a reduction in mortality on the waiting list[8]. However, the score suffers from several limitations: Patients with cholestatic liver diseases, severe portal hypertension as well as those with hepatic encephalopathy are disadvantaged due to a preserved synthesis function which does not reflect the severity of the disease and the related morbimortality. Another major limitation is the absence of assessment of malnutrition and more specifically of sarcopenia[8-10]. Abnormalities of body composition are frequently found in patients with chronic diseases such as cirrhosis. Interestingly, there is a close relationship between the skeletal muscle compartment and liver function (Figure 1)[11].

In the field of chronic liver disease (CLD) and particularly ESLD and LT, different concepts have emerged and are commonly used, such as sarcopenia, malnutrition, frailty or myosteatosis[12]. They affect between 40%-70% of cirrhotic patients with important clinical outcomes. They have been evaluated independently in different studies and it is confirmed that they are robust predictors of poor outcomes[13]. This fragile population is at increased risk of hepatic decompensation, reduced quality of life, increased risk of infection, and prolonged hospitalization. It leads to a higher morbidity and mortality[14,15]. Adding the concept of frailty on the waiting list prioritization, specifically helps a population that is listed with low priority based on a low MELD score (< 15). While adding muscle surface to the MELD score had limited added value for organ allocation in a global cirrhotic population, this measurement is highly correlated with waiting list mortality in patients with low MELD score (< 15) [16]. It is therefore important to define the best tools to evaluate these functional concepts in the field of liver diseases and LT[13].

Understanding these different concepts and their implications in our clinical practice is of major interest to improve the management of our patients. There is clearly a certain overlap in these definitions, but it is important to understand them separately to develop tailored behavioral interventions and targeted pharmacotherapies for these conditions[17]. It is necessary to clearly define the gold standard of sarcopenia management with standardized cut-offs for this specific cirrhotic population. These clear definitions will allow us to evaluate the impact of interventions on patients' outcome[13]. We then review the pathophysiology of cirrhosis-related sarcopenia and myosteatosis, provide a narrative review of the major studies on the subject and discuss the tools available for muscle assessment and treatment.

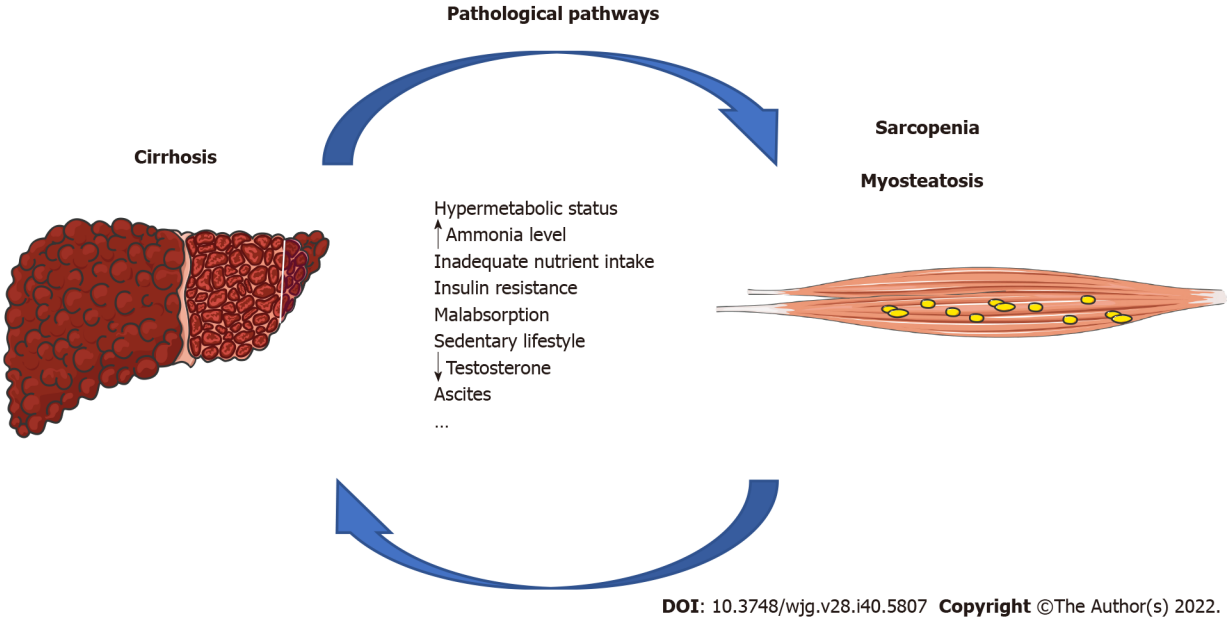

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Figure 1 Summary of pathways linking sarcopenia and myosteatosis to cirrhosis: A bi-directional communication. This figure was partly created using Servier Medical Art templates (https://smart.servier.com).

DEFINITIONS OF COMMONLY USED YET DISTINCT CONCEPTS

As explained above, various terms are now frequently used in the field of CLD, sometimes incorrectly or inappropriately. However, they each have a precise definition.

Malnutrition

Malnutrition can be defined as a state resulting from an insufficient intake of nutrients or an imbalance in essential nutrients and/or in their utilization. This imbalance will have a deleterious impact on the body composition and body cell mass that could lead to diminished physical and mental function, as well as impaired clinical outcomes from disease. Malnutrition has multiple causes combined or take alone, which are starvation and diseases such as CLD and aging[18]. It is also important to note that malnutrition can affect the entire body mass index (BMI) spectrum from obese to anorexic[17]. Different clinical scores exist to estimate the presence of malnutrition such as the nutritional risk score or the Global Leadership Initiative on Malnutrition criteria. Malnutrition is associated with increased mortality in cirrhotic patients[19].

Sarcopenia

The term sarcopenia comes from Greek origin and literally means the "loss of flesh". Sarcopenia is characterized by 3 components: Decreased muscle mass, decreased muscle strength as well as decreased physical performance[20]. Initially, sarcopenia was described as associated with aging. The term is now also used in association with chronic diseases such as cirrhosis[12]. Nevertheless, even today, the criteria to be included as well as the cut-off values are still discussed. The diagnosis of sarcopenia is still complicated today as the criteria can be so variable, especially in obese people[21]. Cutoffs for muscle mass depletion exist but are still currently under debate. Nevertheless, the use of muscle area measurement at the level of the third lumbar vertebra on a computed tomography (CT) slice is recognized as acceptable by relating the muscle area to the patient's height squared [skeletal muscle index (SMI)]. By extension, the term sarcopenia is often used based on the sole criterion of low muscle area. Sarcopenia is then defined when SMI is < 41 cm^2/m^2 for a woman and < 53 cm^2/m^2 for a man in the field of obesity[22] or < 39 cm^2/m^2 for a woman and < 50 cm^2/m^2 for a man in the field of ESLD[23]. In the field of liver diseases, this measure is particularly interesting because it is minimally affected by hydro-sodium retention. Measurement of the brachial circumference (BC) or mid-arm muscle circumference and arm strength using the hand dynamometer are other frequently used techniques.

Myosteatosis

Myosteatosis can be defined as the ectopic accumulation of fat in the muscle. This increased accumulation around and within the myocyte can lead to an impairment of normal physiological function. We will therefore speak of intra and extra cellular fat. It can be estimated on the basis of the decrease in muscle density on an ultrasound image or a CT scan section for example[24,25]. A skeletal muscle radiation attenuation below 41 Hounsfield units (HU) in normal weight patients (BMI up to 24.9 kg/m^2)

and 33 HU in overweight patients (BMI ≥ 25 kg/m²) could be used[24]. Muscle density can also be related to the muscle surface to give an idea of the absolute amount of intramuscular fat[26]. This leads to the following parameters: The skeletal muscle density index (SMDI)[27] and the skeletal muscle fat index (SMFI) described in patients with MAFLD without ESLD[26,28]. Different groups of patients are compared in clinical studies, but pathological cut-offs for SMDI or SMFI are not yet defined[26-28]. Finally, imaging allows us to assess the homogeneous or heterogeneous distribution of fat in the muscle. This parameter can be associated with certain pathological conditions[29].

Frailty

Defined in the field of geriatrics, frailty is considered a metabolic syndrome associated with a decrease in physiological reserves and an increase in vulnerability to stress factors. In the case of cirrhosis, the stressors are variable, ranging from a dysfunction in protein synthesis to muscle toxicity. A reproducible and objective assessment of patients with liver disease is offered by several simple, non-invasive tests. For example, based on the results of handgrip, sit to stand and balance tests, the liver frailty index (LFI) can be calculated. A patient is considered frail if the score is between 4.5 and 6[15].

Physical exercise

Physical exercise is defined as activity requiring physical effort, performed specifically to maintain or improve health and fitness. The term is therefore relevant to physical activity interventions offered to patients with CLD to improve their condition. The data available so far concerns patients with compensated liver disease with no separate results for patients with ESLD[30]. An increase in functional capacity, strength and muscle mass is noted in the patients with compensated cirrhosis who are subjected to physical exercise for 12 wk and then compared to a group of patients with relaxation activity[31]. In patients awaiting LT, specific protocols are being evaluated (NCT04604860, NCT05061576, NCT05237583, *etc*). The type (endurance and/or resistance), duration and frequency should be determined, bearing in mind that these programs must be adapted to the patients' situation (safety, absence of contraindications).

PATHOPHYSIOLOGICAL BASIS OF SARCOPENIA AND MYOSTEATOSIS IN END STAGE LIVER DISEASE

Several mechanisms exist that link sarcopenia to CLD and ESLD. Nevertheless, these exact mechanisms are particularly not well known. Sarcopenia is often a feature of malnutrition. The reasons for this malnutrition, in the case of cirrhosis, are multiple and often result from the combination of several factors (Figure 1)[32]. The first factor in the context of CLD and cirrhosis is the presence of a hypermetabolic status resulting from chronic inflammation and altered gut barrier function[33]. Low liver glycogen content induces muscle protein catabolism for blood glucose maintenance[34]. In addition, ascites present in cirrhotic patients may be responsible for an increase of energy expenditure and therefore increase the catabolism of protein[35]. Partly due to the pro-inflammatory environment, patients with cirrhosis and HCC showed accelerated sarcopenia[36]. Second, the hyperammonemia level is also increased due to its poor elimination because of a pathological architecture of the liver. The consequence of this increase will be the activation of myostatin and consequently the inactivation of protein synthesis which will lead to sarcopenia. Third, inadequate intake of micro and macronutrients can be due in cirrhotic patients to loss of appetite (dysgeusia, cytokines), prolonged gastric emptying or a restrictive diet (sodium and protein) for example. The sedentary lifestyle is probably the most obvious factor in sarcopenia. Indeed, a study showed that one of the causes of sarcopenia in the elderly was partly due to lack of physical exercise[20]. It is therefore easy to think that decompensated cirrhotic patients with high frailty and malnutrition may also suffer from a lack of physical activity aggravating their sarcopenia even more. Fourth, cirrhosis is associated with some degree of cholestasis. An increase in bile acids related to deregulation of the enterohepatic cycle (also due to the dysbiosis associated with cirrhosis) is observed. These have a potentially toxic effect, particularly on the muscle that expresses some bile acid receptors, inducing muscle atrophy[37]. Malabsorption in the context of cirrhosis also plays an important role. A decrease in bile excretion will result in a decrease in the reabsorption of lipids and certain lipo-soluble vitamins. Altered intestinal motility as well as changes in the composition of the gut microbiota will also affect nutrient absorption and utilization[33]. Finally, in cirrhotic patients, a decrease of testosterone levels has been associated with a decrease of muscle mass that could favor sarcopenia[34].

The mechanism through which myosteatosis occurs is still not well understood. Myosteatosis is a well-known characteristic of patients with CLD due to MAFLD[27]. Cirrhotic patients are also characterized by muscle insulin resistance, regardless of the cause of the cirrhosis[34]. This insulin resistance status may also be responsible for myosteatosis[34]. Other proposed mechanisms include hyperammonemia (inducing impaired mitochondrial oxidative phosphorylation and decreased lipid oxidation in muscle), decreased lipid storage capacity within the subcutaneous adipose tissue and age-related differ-

entiation of muscle stem cells into adipocytes[38].

WHY IS IT IMPORTANT TO CONSIDER THE MUSCLE IN A LIVER TRANSPLANT SETTING?

Screening and preventing sarcopenia and frailty is of major interest to patients with CLD. The challenge is big because muscle composition and muscle function have a direct impact on the prognosis of the cirrhotic patient. Sarcopenia and frailty will condition the prognosis of the cirrhotic patients awaiting LT, independently of the severity of the liver disease (MELD score)[39]. Table 1 summarizes the main studies investigating the deleterious role of sarcopenia, frailty and myosteatosis in the context of LT.

For more than a decade, many prospective studies have demonstrated that low muscle mass is a major predictive factor of mortality in the waiting list but also after LT. In a study involving more than 200 patients on the waiting list, both low muscle quantity and quality were independent risk factors for mortality in patients with ESLD[40]. Low muscle mass is more frequent in patients with hepatic encephalopathy and associates with poor survival[41]. Two meta-analyses confirm these results, the first one includes more than 19 studies with 3800 patients awaiting LT. Sarcopenia was assessed by a wide range of CT-assessed skeletal muscle mass cut-points. Despite these limitations, the study described an independent association between low muscle mass and the post-LT and the waiting list mortality[42]. The second meta-analysis, although it has multiple biases, shows an association between sarcopenia in the pre-LT period and greater post-operative mortality, higher infection risk and post-operative complications, longer intensive care unit stay and ventilator dependency[43].

In patients with cirrhosis (decompensated or not), frailty, easily measured by the LFI, is an independent predictor of death, cirrhosis progression and unplanned hospitalizations (mainly due to infections)[44]. Frail cirrhotic patients also have an increased risk of acute kidney injury[45] and hepatorenal syndrome[46]. In cirrhotic patients awaiting LT, frailty is associated with a 2-fold increased risk of mortality, regardless of the presence of ascites or hepatic encephalopathy[15]. Frail cirrhotic LT recipients have increased postoperative morbidity with prolonged hospital stays[47] and an increased risk of acute rejection[48].

While the link between sarcopenia and mortality is well established, the long-term prognostic value of skeletal muscle quality is less clear. However, several studies tend to demonstrate that pre-transplant myosteatosis is an important prognostic marker for LT recipients in the post-operative period. The prognosis value of myosteatosis seems to be particularly important in the early post-operative phase with higher rates of deaths due to respiratory and septic complications[48]. Unfortunately, most studies are retrospective and the clinical impact of myosteatosis on the transplant waiting list still needs to be demonstrated with prospective studies.

In view of these results, the American Association for the Study of Liver Diseases guidance document suggests that all cirrhotic patients should be assessed for sarcopenia and frailty with standardized tools both at baseline and longitudinally[17]. These recommendations are consistent with the implementation of specific protocols such as "enhanced recovery after surgery" protocols to improve recovery after surgery, especially for LT candidates[49].

HOW TO EVALUATE THE MUSCLE IN ROUTINE PRACTICE?

To assess physical activity, muscle function, composition and mass, several tools exist. It is important to note that many tests, questionnaires and radiological methods are possible. A non-exhaustive list of these tools is presented in Table 2 with their advantages and disadvantages. Some of them have been included in a recent review manuscript[50]. It is interesting to note that patients' baseline physical activity can be assessed by questionnaire or objective measurement (Table 2). Easy tests are possible in consultation or at the patient's bedside, such as measuring BC or handgrip strength. Tests that are more complex to implement in routine practice are also available, such as the 6 min walk test, which correlates well with survival in patients awaiting LT[51], or isokinetic testing, the detailed results of which have given interesting information in other diseases such as type 2 diabetes[29]. Finally, among imaging techniques, the same dilemma exists between easier and less expensive techniques (such as ultrasound)[52,53] and time-consuming but probably more objective and reproducible techniques (such as CT or magnetic resonance imaging)[54] (Table 2).

HOW TO IMPROVE THE MUSCLE IN CLINICAL PRACTICE?

It is well defined that malnutrition plays a major role in the prognosis of the cirrhotic patient and that the resulting loss of muscle mass is a real complication of cirrhotic disease with a significant impact on the morbidity and mortality of these patients. All patients with CLD should be evaluated for nutrition

Table 1 Main studies investigating the deleterious role of frailty, low muscle mass, sarcopenia and myosteatosis in patients undergoing pre-transplant assessment

Ref.	Patient selection	Patients, *n*	Sarcopenia, myosteatosis	Frailty parameters	Outcome	Results
Lai *et al* [15], 2019	Ambulatory cirrhotic patients awaiting LT	1014	Non applicable	LFI	Waiting list mortality	Frailty: 25%. HR = 1.82; 95%CI: 1.31-2.52; P < 0.001
Fozouni *et al*[48], 2020	LT recipients	241	Non applicable	LFI	Acute cellular rejection within 3 mo post-LT	Frailty: 19%. OR = 3.3; 95%CI: 1.2-9.3; P = 0.02
Lai *et al* [47], 2022	LT recipients	1166	Non applicable	LFI	LT LOS. ICU stay. Inpatient days within 3 mo post-LT. Overall survival (1-3 and 5-yr mortality)	Frailty: 21%. Prolonged LT LOS: OR = 2.00; 95%CI: 1.47-2.73. ICU stay: OR = 1.56; 95%CI: 1.12-2.14. Inpatient days within 3 mo. post-LT: OR = 1.72; 95%CI: 1.25-2.37. Overall survival: P = 0.02
Bhanji *et al*[41], 2018	Cirrhotic patients assessed for LT	675	L3-MA (HU). L3-SMI	Non applicable	HE. Mortality	Sarcopenia: 36%. HE: OR = 2.42; 95%CI: 1.43-4.10; P = 0.001. Mortality: Univariate HR = 2.02; 95%CI: 1.57-2.58; P < 0.001. Multivariate: HR = 2.15; 95%CI: 1.52-3.05; P < 0.001. Myosteatosis: 52%. HE: OR = 2.25; 95%CI: 1.32-3.85; P = 0.003. Mortality: Univariate: HR = 1.45; P = 0.004; 95%CI: 1.16-2.91. Multivariate: P = 0.69
Van Vugt *et al*[42], 2016	LT candidates/recipients	3804. Meta-analysis	SMI. Total psoas area	Non applicable	Waiting list mortality. Post-LT mortality	Waiting list mortality: HR = 1.72; 95%CI: 0.99-3.00; P = 0.02. Post-LT mortality: HR = 1.84; 95%CI: 1.11-3.05; P = 0.05
Bot *et al* [40], 2021	ESLD patients awaiting LT	262	L3-SMI (cm^2/m^2). L3-MA (HU)	Non applicable	Waiting list mortality	Low SMI: HR = 2.580; 95%CI: 1.055-6.308. MA: HR = 9.124; 95%CI: 2.871-28.970
Czigany *et al*[24], 2020	LT recipients	225	L3-SMI. L3-MA (HU)	Non applicable	Major morbidity. ICU stay. LT LOS	Sarcopenia: 37%. Myosteatosis: 44%. Major morbidity: OR = 2.772; 95%CI: 1.516-5.066; P = 0.001. ICU stay: 18 ± 25 *vs* 11 ± 21 d, P < 0.001. LT LOS: 56 ± 55 *vs* 33 ± 24 d, P < 0.001

LFI: Liver frailty index; HR: Hazard ratio; CI: Confidence interval; LT: Liver transplantation; OR: Odds ratio; LT LOS: Liver transplantation length of stay; ICU: Intensive care unit; L3-MA: Lumbar 3-muscle attenuation; HU: Hounsfield unit; L3-SMI: Lumbar 3-skeletal muscle index; HE: Hepatic encephalopathy; SMI: Skeletal muscle index; ESLD: End stage liver disease.

and sarcopenia at the time of diagnosis and then regularly at least annually for compensated cirrhosis and every 8-12 wk for decompensated cirrhosis[17]. The ultimate goal of the medical practitioner is the prevention of the occurrence of complications that may result from sarcopenia and undernutrition. Nevertheless, although there is no clear evidence for an appropriate guideline for patients at present, the current management in transplant centers is that patients with cirrhosis who are placed on the list benefit from efforts by the multidisciplinary team to try to preserve muscle mass and function, through the interventions of dieticians (screening for undernutrition, implementation of standard nutritional measures, early oral intake) and physical therapists (mobilization)[54]. However, it is not yet proven that we are able to slow down the progression of complications or reverse advanced situations *via* muscle targeted interventions[13]. There are two main options, non-exclusive: Dietary interventions and physical activity. Additional specific pharmacological measures are under investigation.

The current nutrition guidelines for patients with CLD recommend a weight-based daily caloric intake of at least 35 kcal/kg/d with a protein intake of 1.2-1.5 g/kg/d[18]. These recommendations are derived from data assessing energy expenditure (indirect calorimetry). They are difficult to achieve in routine practice. Protein intake is easily found in staple foods such as meat and vegetables. Several studies have analyzed the benefit of branched-chain amino acid (BCAA) supplementation but the results of these data are contradictory. While some authors suggest that BCAA improve hepatic encephalopathy and muscle mass[55], a meta-analysis shows that there is no effect on mortality and nutritional parameters[56]. Because of these contradictions and the limitations of clinical studies, BCAA supplementation is not currently recommended. Prolonged periods of fasting should be avoided in cirrhosis and it is important to favor split meals associated with a late evening snack or an early morning breakfast[17]. Oral nutritional supplements given before bedtime showed a muscle benefit in Child A patients but not Child B or C[57]. If not achieved *via* oral route, energy intake *via* enteral nutritional supplementation may be considered to achieve targets.

Besides these nutritional recommendations, physical activity improvement is also important. The latest recommendations propose to assess frailty and/or sarcopenia with standardized tools in order to define a personalized approach for the sarcopenic patient. This personalized activity prescription is

Table 2 Set of techniques to evaluate the physical activity as well as the quantity and quality of muscle

Technique	Goal	Advantages	Disadvantages
Physical activity assessment			
IPAQ	Reflects physical activity over the last 7 d	Easier to implement. Understandable for the patient. Representative over 7 d	Subjective. Poor estimation by the patient
Pedometer	Measures walking activity	Inexpensive and easy to set up. Represents the physical activity of walking	Reliability decreases with walking speed. Could induce changes in patient's habits. Does not measure other physical activities
Accelerometer	Measures walking activity	Takes slope and weight carried in consideration	Long to wear for good representation. Not sensitive to the inclination of the walk or the load worn during the walk. Could induce changes in patient's habits
Anthropometric measurement			
BC	Gives information on arm circumference and stage of muscle mass depletion	Very easy to perform (requires only a tape measure)	User dependent
MAMC (BC-TSF)	Gives information on arm muscle volume	Well described and easy to perform. Assesses muscle volume better than BC alone	User dependent. Requires a caliper
BIA	Allows to assess the body composition	Simple. Non-invasive. Reproductible	Requires specific equipment. Varies with fluid retention (common in cirrhosis)
Function tests			
Handgrip	Allows to measure the strength of the forearm and wrist muscles	Low cost. Easy to perform. Gold standard	Not representative of total body strength
6MWT	Measures distance walked for 6 min	Easy to perform. Reflects endurance and general fitness	Caution in patients with cardiac and respiratory problems
LFI	Measures frailty of patients calculated with a score	Easy to perform. Little training	Not assessable in severe encephalopathy
Isokinetic	Measures the function and strength of a of group muscles	Gold standard. Reproducible	Cost. Requires training and collaboration. Caution in patients with cardiac and respiratory problems
Leg press	Measures the strength of the lower limbs	Simple device	Requires collaboration. Rougher assessment. Not well evaluated/used
Imaging techniques			
US	Allows to measure the echogenicity of the muscle	Non-invasive. Not expensive	User dependent. Variability with fat thickness, position of the probe. Requires training
CT-scan	Allows to measure the surface of the muscle (easily) as well as its density	Clear image. Reproduceable	Radiation exposition
MRI	Can be used to evaluate the muscle surface. Measures the amount of fat within the muscle (PDFF)	Clear image. Reproduceable	Cost. Time consuming. Contra-indications (claustrophobia, pacemaker, *etc*)
DXA	Allows to measure the body composition (muscle, fat, water, *etc*)	Clear image. Reproduceable	Radiation exposition. Varies with fluid retention (common in cirrhosis)

IPAQ: International physical activity questionnaire; BMI: Body mass index; BC: Brachial circumference; TSF: Tricipital skin fold; MAMC: Mid-arm muscle circumference; BIA: Bio-impedance analysis; 6MWT: 6 min walk test; LFI: Liver frailty index; US: Ultrasound; CT-Scan: Computed tomography scan; MRI: Magnetic resonance imaging; PDFF: Proton density fat fraction; DXA: Dual x-ray absorptiometry.

guided by the principles of frequency-intensity-time-type: A combination of aerobic exercise that improves cardiorespiratory endurance and resistance exercise that improves strength and skeletal muscle mass[17]. Personalized physical activity allows patients to improve their cardiopulmonary capacity and quality of life. Unfortunately, current randomized controlled data are limited by small samples of well-compensated cirrhosis patients (mean MELD = 10)[31,58]. Despite these promising results, there are no randomized controlled studies with patients with ESLD or a waiting list for LT. It is therefore not currently possible to conclude whether a personalized physical activity program would improve the outcomes in the waiting list and post-LT surgery.

Several randomized controlled trials evaluating the benefit of nutritional and/or physical exercise on muscle mass (SMI) and frailty (LFI) pre- and post-LT would help to answer these uncertainties. Finally, pharmacological treatments are being studied to treat sarcopenia (hormone replacement therapy such as testosterone or growth hormone, ammonia reduction and myostatin inhibition)[59].

CONCLUSION

Malnutrition, sarcopenia and frailty are very common in patients with cirrhosis, especially in cases of disease requiring organ transplantation. Bi-directional pathophysiological links exist between muscle and liver that underlie this association. This highlights the interest of prospective studies comparing the different tools at our disposal to evaluate the cirrhotic patient as well as translational studies to understand these mechanisms and find a possible therapeutic or diagnostic target. Through a better understanding of mechanistic links, the muscle can become a game changer. A better understanding of the situation will also allow us to prioritize fragile patients who need a faster LT or to identify negative factors in certain recipients in whom the situation would be irreversible post-LT and deleterious to the graft. Interventional strategies (nutritional, physical exercise and pharmacological treatments) are also being evaluated. The rationale for their efficacy is clear, even if at this ESLD, evidence of their impact on reducing pre-LT and post-LT mortality is lacking, as well as a precise characterization of the protocols to be implemented. Again, quality prospective studies will be able to answer this question.

FOOTNOTES

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Abstract

There is increasing incidence and prevalence of acute and chronic liver diseases (CLDs) all over the world which influence the quality of life and can give rise to life threatening complications. The burden of advanced liver disease due to hepatitis B has been controlled by antivirals but its eradication is difficult soon. Highly effective directly acting antiviral therapy has reduced the burden of hepatitis C but is partially offset by increasing IV drug abuse. Non-alcoholic fatty liver disease pandemic is on and there is recent alarming increase in alcohol related liver disease, both of which have no drug cure apart from control of the risk factors. Genetic factors have been identified in progression of all forms of CLD. Due to better management of complications of CLD, the life span of patients have increased spiking the number of hepatocellular carcinoma (HCC) and patients needing liver transplantation (LT). The present severe acute respiratory syndrome coronavirus pandemic has affected the outcome CLD including LT in addition to causing acute hepatitis. Better diagnostics and therapeutics are available for liver fibrosis, portal hypertension, HCC and post LT management and many drugs are under trial. The present review summarises the current scenario of the epidemiology and the advances in diagnosis and treatment of liver diseases including their complications like portal hypertension, HCC and LT.

Key Words: Chronic liver disease; Genes; Biomarkers; Therapy; Hepatocellular carcinoma; Liver transplantation; Recent advances

Core Tip: The incidence and prevalence of liver disease is rising all over the world. Hepatitis B is difficult to eradicate and the benefit of directly acting antiviral therapy for hepatitis C is partially offset by increasing IV drug abuse. Non-alcoholic fatty liver disease pandemic is on and alcohol related liver disease is rising alarmingly, both having no drug cure. Due to better management of complications, patients of chronic liver disease are living longer spiking the number of hepatocellular carcinoma (HCC) and patients needing liver transplantation (LT). Better diagnostics and therapeutics are available for fibrosis, portal hypertension, HCC and post LT management which are discussed.

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INTRODUCTION

Chronic liver disease (CLD) and cirrhosis pose substantial health burden worldwide. In the period 2007-2017, the age standardised prevalence increased 10.4% with 1.5 billion cases in 2017[1]. Of the four chief etiology, hepatitis B virus (HBV) and hepatitis C virus (HCV) burden still remains high [though decreased due to availability of vaccination for HBV and directly acting antiviral therapy (DAA) for HCV] although with the non-alcoholic fatty liver disease (NAFLD) pandemic and increasing global alcohol consumption, they are fast catching up. NAFLD is the leading cause in developed nations, it is also gradually becoming important in newly developed nations like India, China[2,3]. The age standardised prevalence of HBV/HCV related CLD rose by 9%/10.2% in the last decade whereas for NAFLD it was 23.5%[1]. The high HBV and HCV burden is mostly due to poor diagnostic coverage and linkage to treatment and care of the susceptible population.

HBV

The HBV pool is chiefly contributed to by the Western Pacific and Subsaharan Africa region (mostly tribals) and some southeast Asian countries (China, Vietnam, Thailand, Laos) where the load remains high despite the success of HBV vaccination programme at birth. It is the leading cause of hepatocellular carcinoma (HCC) in these countries[4]. Among some developed and newly developing nations where prevalence is intermediate to low, its burden is contributed by the indigenous tribal population like India, Australia[5,6] maintained through intracaste marriages, close living, tribal customs, illiteracy and poor access to health care resources. With the present attrition rate (present burden of 296 million from 350 million 3 decades back and present annual mortality of 8 lakh and addition of 1.5 million cases in 2019[7]), it is still a long way for natural elimination of the pool. In future some redistribution is also likely due to population migration from high to low endemicity regions. World Health Organization (WHO)'s ambitious programme for eradication of HBV by 2030 therefore incorporate the best preventive measures *i.e.*, increase vaccination at birth, prevent vertical and horizontal transmission among toddlers by treating at risk mothers, and scale up screening, care and treatment services. Curative treatment is difficult and < 20% who receive the currently approved drugs [interferon, nucleos(t)ide analog (NA) or combination as sequential/add on/switch therapy] achieve loss of HBsAg (functional cure). Combination strategies are less cost effective than first line NA monotherapy although this may lead to more HBsAg loss in some subgroup of HBV patients[8]. Even with long term NA monotherapy (Tenofovir disoproxil for 5 years) half fail to achieve fibrosis regression[9] and there is high relapse rate in e negative patients (RETRACT B study showing relapse rate of 47.8% at 6 mo, 68.9% at 12 mo, 83.4% at 48 mo)[10]. The other problem is the risk of relapse in previously exposed person or inactive HBsAg carriers (who constitute a sizable majority of the present pool not requiring drug therapy) needing immunosuppression (IS) or cancer chemotherapy. Fortunately, highly active antivirals are capable of controlling the virus and reducing the burden of advanced liver disease from HBV. The chief impediments to HBV functional cure are intrahepatic viral reservoir cccDNA with integrated sequencing, high HBsAg levels, and defective host innate and adaptive immune responses. Newer strategies target these *e.g.*, targeting HBV life cycle without damaging hepatocyte by inhibiting ccc DNA replenishment pathways or degrading them by entry inhibitors like Bulevirtide [used for HBV/HDV coinfection including post liver transplantation (LT)], nucleic acid polymer assembly inhibitors (Lonafarnib), CRISPR/Cas9 protein base editors (DNA endonucleases), siRNAs, core protein modulators (Morphothiadin,Vebicorvir, Bersacapavir) and antisense oligonucleotide (Bepirovirsen)[2], immunomodulation to safely eliminate infected cells. Potential targets in innate immune response pathway include pathogen recognition receptors [Toll-like receptors 7/8, retinoic acid-inducible gene

(RIG)-1-like receptors and nucleotide-binding oligomerization domain (NOD)-like receptors], natural killer cells and antigen presenting cells (dendritic cells and Kupffer cells) whereas in adaptive immune response pathway, it includes modulation of HBV-specific CD4+ and CD8+ T cell (especially the relative functional and numerical deficiency of CD8+ T-cells by PD-1 checkpoint inhibitors), regulatory T cell, HBV-specific T and B cell (autologous, engineered or by vaccine).

HCV

Gratifying results have been obtained with the introduction of affordable short term (3-6 mo) DAA therapy for HCV (with sustained viral response rates of > 95%, decreased fibrosis and HCC) with increasing treatment coverage in newly developed and developing nations which have decreased the HCV burden to 58 million as of 2019[7]. HCV still remains the leading cause of HCC in the developed world (Western countries, Japan) though alcohol related liver disease (ALD)/NAFLD are fast taking the lead due to treatment with DAA. But challenges still remain like limited drug availability, interaction with other drugs used to treat comorbidities (HIV, coronary artery disease and hyperlipidemia), inability to afford even the low drug cost by patients who pay from their own pocket and increasingly recognized metabolic dysfunctions associated with hepatitis C. Even in Denmark, 50% HCV patients are yet to attend specialist care especially IV drug users[11]. HIV coinfection is also a deterrent for good treatment outcome for both HBV and HCV. In future the HCV pool is likely to be maintained by intravenous drug users and the increasing population with drug/alcohol abuse and other psychiatric disorders, those needing repeated blood transfusion (for haematological disorders, hemodialysis) and reinfection in those who continue to have risk factors even after cure by DAA. WHO recommends increased access to treatment by onsite diagnosis by dried blood spot and initiating treatment at point of care and by trained non specialist doctors and nurses at harm reduction centres.

NAFLD

NAFLD is the most common liver disease worldwide affecting about a quarter population with regional differences[12]. It is fast becoming the leading cause of cirrhosis in developed nations. Genetic inheritance (25%-34%), ancestry (HispanicAmerican/Asian/Indian > European > African American), advancing age and male sex are non modifiable whereas obesity (especially central), diabetes mellitus, hyperlipidemia and insulin resistance are modifiable risk factors. There is currently no approved pharmacological therapy for NAFLD apart from those treating the risk factors. Weight loss through dietary alteration, physical exercises and bariatric surgery leads to improved liver histology but only small percentage of patients can achieve and maintain the degree of weight loss needed for sustaining the benefit and 50% fail to improve histology[13]. Ursodeoxycholic acid (UDCA)/obeticholic acid (OCA), Vitamin E have no proven benefit. Therefore it is the hottest area of newer drug research which modulate key metabolic, inflammatory, and fibrogenic pathway. Pan PPAR agonists (Lanifibranor), GLP 1 agonists (Semaglutide), CCR 5 inhibitors (Leronlimab), thyroid hormone receptor agonist (Resmetirom) and hepatic SCD1 inhibitor (Aramchol) are in phase 2 and 3 clinical trial (Table 1). Other antifibrotic and disease modifying agents as well as genetic factors are discussed below. But considering the multiple risk factors and complex pathophysiology, it is unlikely that a panacea will be discovered soon.

ALCOHOL

Approximately 2 billion people worldwide consume alcohol of whom 283 million suffer from AUD[14]. ALD is most prevalent in the western world and in some affluent Asian countries (South Korea, Japan) though there is increasing global trend especially in newly industrialised southeast Asian nations (China, India, Vietnam, Thailand) where it was low due to traditional"dry" culture. ALD has become the leading cause of CLD/cirrhosis in India[5]. The recent coronavirus disease 2019 (COVID-19) pandemic has significantly increased the incidence of ALD in young adults. DALYs per 1000 people due to ALD was highest in India (2356.4), followed by the United States (467.9), China (466.3), Nigeria (424.5) and Indonesia (365.1). For alcohol related liver cancer, DALYs were highest for China followed by Vietnam, Russia, Thailand, India[15]. Consumption depends on age, sex, religion, culture, health status and national income distribution. Globally it is a tussle between national income from alcohol retail *vs* health expenditure for AUD, the latter being dismal even in developed nations. Being a fully preventable disease, WHO's "best buys" are the most cost effective ways for prevention, *i.e.*, increasing taxation on alcoholic beverages, enforcing bans or comprehensive restrictions on exposure to alcohol advertising and restricting physical availability of retailed alcohol. A recent global study shows no safe dose for alcohol[16]. Abstinence can reverse fatty liver and halt the progression of CLD. It is responsible

Table 1 Interrim results of selective drug trials for non-alcoholic fatty liver disease and liver fibrosis

Agent	Mechanism	Phase	ClinicalTrials.gov number	Results
Simtuzumab	Lysyl oxidase-like 2 monoclonal antibody	IIb	NCT01672879	Ineffective in decreasing hepatic venous pressure gradient
Selonsertib	Selective inhibitor of apoptosis signal-regulating kinase 1	III	NCT03053063	Ineffective in improving fibrosis without worsening NASH
Emricasan	Pan-caspase inhibitor	II	NCT03205345	No reduction in composite outcome of mortality and decompensation
Pegbelfermin	PEGylated fibroblast growth factor 21 analogue	IIa	NCT03486912	Ineffective in improving fibrosis without worsening NASH
Lanifibranor	Pan peroxisome proliferator-activated receptor agonists	III	NCT04849728	Decrease of ≥ 2 points in the Steatosis Activity Fibrosis score without worsening of fibrosis in phase 2b trial
Resmetirom	Thyroid hormone receptor agonist	III	NCT03900429	Significant reductions in liver fat content and serum atherogenic lipids in phase 2 trial
Aramchol	Hepatic stearoyl-CoA desaturase1 inhibitor	IIb	NCT02279524	Insignificant decrease in liver triglycerides but significant improvement in liver inflammation and improvement of fibrosis ≥ 1 stage and serum ALT level
Leronlimab	Chemokine receptor 5 monoclonal antibody	II	NCT04521114	Significant drops in liver fat, inflammation and fibrosis values as also in liver enzymes and multiple inflammation markers at week 14 compared to placebo

NASH: Non-alcoholic steatohepatitis; ALT: Alanine transaminase.

for 50% of deaths due to CLD because no specific drug therapy is available apart from some short term benefit of steroids in pure acute hepatitis. Tumour necrosis factor alpha, growth hormone, pentoxyfylline and antioxidants at best show mixed results from highly variable to weak, efficacy depending on the stage of disease. The unclear molecular mechanism of disease deter identifying treatment target and disincetivize drug development. Naltrexone, disulfiram and acamprosate helps to decrease addiction. Obesity and cigarette smoking are known risk factors so weight control and quitting smoking are routinely encouraged. A poor overall nutritional status (protein calorie malnutrition, micronutrient deficiencies) often accompanies ALD and correlates positively with the development of serious complications hence a well-conceived nutrition support by oral, enteral, and parenteral routes is an essential part of standard care. Recent evidence also strongly implicates intestinal dysbiosis in ALD progression. These targets are being addressed by trials of probiotics, fecal microbiota transplantation, growth factors (granulocyte colony stimulating factor, bovine colostrum), antioxidants (ω5 and synthetic fatty acids, S-adenosyl methionine + choline, N-Acetyl cysteine, vitamin C), in addition to liver regenerative biologics and device assisted behavioural alteration[17]. The other hindrances are disease stratification for early identification when it is most reversible, monitoring abstinence (as recidivism is high) and identifying risky drinking behaviour like binges. Various biomarkers under study for this purpose include circulating small noncoding RNAs, long noncoding RNAs, selective cytokines profiles, phosphatidyl ethanol and urine ethyl glucuronide and ethyl sulphate[18-21].

AUTOIMMUNE LIVER DISEASE

Autoimmune hepatitis appears to be increasing in incidence as a part of the general increase in immune mediated and allergic diseases resulting from decreasing infectious disease with mounting antibiotics use globally. Some antibiotics like nitrofuranotin, minocycline and coamoxyclav can induce autoimmune hepatitis by themselves and some antibiotic associated drug induced liver disease (DILI) may resemble autoimmune hepatitis. The standard treatment of autoimmune hepatitis is with steroids with/without azathioprine. Mycophenolate mofetil is a second line drug. Substantial advances in treatment of autoimmune cholangiopathies has been achieved with PPAR α agonist bezafibrate, FXR agonist OCA and recombinant FGF 19 (which alter bile acid synthesis along with antifibrogenic effect [22], see below) and drugs inhibiting intestinal apical sodium-dependent bile acid transporter (linerixibat, maralixibat, odevixibat)[23]. in addition to bile acid resins and UDCA. Combinations of such enterohepatic with cholehepatic and/or anti-fibrotic drugs could result in synergistic/additive effects in decreasing the fibrosis along with the pruritus.

ADVANCES IN DIAGNOSIS

Non invasive biomarkers of CLD (patented ones like fibrotest, fibrometer, Hepascore, ELF model and non patented ones like FIB 4 index, APRI, BARD, NFS) and elastography (fibroscan, MRE, point SWE, 2D-SWE, 3D Velacur™) or their combination (MEFIB, MAST, FAST) have been investigated across the whole spectrum of NAFLD to delineate the stage as well as correlating genes with liver fat, enzymes and fibrosis[24]. Novel ones like computerised tomography (CT) scan with objective measures of liver nodularity and shunts, multiparametric magnetic resonance imaging (MRI) (iron corrected T1, cT1), extracellular vesicles, microbiome (stool microbial profiles), biomarker for extracellular matrix remodelling (TGF-β, MMP, TIMP)[25-27] are being investigated. Graph convolution networks (a deep learning technique) is being tested for quantitative assessment of fibrosis[28].

GENETIC FACTORS IN CLD

Genetic factors are important in progression of all forms of CLD (ALD, NAFLD, metabolic associated fatty liver disease, chronic hepatitis) including HCC with interplay of genes involved in glucose, lipid and iron metabolism, insulin signalling, oxidative stress, inflammatory pathways and fibrogenesis. Most reliable fatty liver genes include PNPLA3, TM6SF2, HSD17B13, GCKR and MBOAT7 (associated with increased liver fat, NASH, cirrhosis, HCC). The evidence for others like MARC1, GPAM, APOE, ALDH1B, PCKS7, SERPINA1, HNF1A etc are less robust. Rare variants like APOB and MTTP are associated with an increased risk of fat accumulation leading to HCC while protecting at the same time against dyslipidemia and cardiovascular risk[24]. Polygenetic risk score [with/without clinical risk markers] are being investigated to stratify disease risk *e.g.*, PNPLA3 and TM6SF2 can become a reason for HCC surveillance whilst giving protection from cardiovascular complications[29] It can also help in proper drug selection. Genetic therapies in CLD include gene silencing approaches (PNPLA3, HSD17B13), CRISPR/Cas9-based approaches[30] (which alter responsible genes) and modulating genes involved in liver regeneration.

ACUTE HEPATITIS

The etiology of acute liver failure (in those with normal liver) varies in different countries at different times. Most commonly these include viruses (hepatitis A, B and E), DILI (CAM, anti tuberculous drugs, paracetamol, anticonvulsants, antibiotics), toxins (herbs, alcohol) and autoimmune flares; else these may precipitate acute liver failure in those with CLD (acute-on-chronic liver failure, ACLF). Hepatitis E virus may be associated with fulminant course in pregnancy. In tropical areas, malaria, dengue, enteric fever, leptospirosis and scrub typhus may also cause acute hepatitis. Traditionally Wilson disease and autoimmune hepatitis has been considered to cause acute liver failure but in adults majority of such acute flares occur on background CLD. Most acute hepatitis of viral etiology recover by themselves and of drug/toxin on their discontinuation, some DILI may need corticosteroid (especially those resembling autoimmune hepatitis). Bacterial infections respond to antibiotics. But the course of ACLF depends on the stage of the background CLD and the precipitating cause, alcohol having the worst outcome[31,32]. Undefined number of acute hepatitis are occurring recently due to COVID-19 infection, recreational drugs and alcohol.

PORTAL HYPERTENSION AND LIVER FIBROSIS

With the increasing prevalence of CLD, portal hypertension and its complications are also increasing. Refractory ascites/hepatorenal syndrome/hydrothorax are now being better managed with terlipressin, noradrenaline, midodrine, octreotide and long-term albumin supplementation (with its newly discovered wider pleiotropic non-oncotic properties positively impacting decompensated CLD). Sodium-dependent glucose cotransporter 2 inhibitors and Alfa pump are under trial. Endohepatology (the application of endoscopic ultrasound in liver disease treatment)[33] has brought about dramatic improvement in the treatment of variceal bleed by better delineation of collaterals and guided treatment (coiling, balloon retrograde transvenous occlusion of collaterals, glue injection in gastric varix), directed liver biopsy, portal pressure gradient measurement and deployment of dedicated esophageal stents. Pre emptive transjugular intrahepatic portasystemic shunt has been used for uncontrollable ascites or variceal bleed.

Fibrosis in the liver is caused by activated HSC whose biology connects damage, regeneration and cancer. Severe hepatic fibrosis represses regeneration and accumulation of senescent HSCs creates a pro-inflammatory, pro-fibrotic environment. Fibrogenesis inhibitor drugs resolve inflammation, cause loss of activated myofibroblasts, and ECM degradation Those under investigation (Table 1) include [FXR

agonist OCA/cilofexor, acetyl-coenzyme A carboxylase inhibitor Firsocostat, ASK-1 inhibitor Selonsertib, CCR 2/5 inhibitor Cenicriviroc, FGF 19 analog Aldafermin/21 analogue Pegbelfermin, PPAR α/δ agonist Elafibranor, PPARγ agonist pioglitazone, PPAR α/γ agonist Saroglitazar, galectin 3 inhibitor Belapectin, CB1 antagonist Rimonabant, ECM production inhibitors like TIMP and MMP, lysyl oxidase 2 inhibitor Simtuzumab, HSP 47 inhibitor Pirfenidone, pan caspase inhibitor Emricasan and anti inflammatory lipids derivatives of PUFA (lipoxins, resolvins, protectins, and maresins)][34-36]. Statins have been found to have beneficial effect in halting the progress of CLD[37].

HCC

The chief cause of HCC in the West is hepatitis C followed by NAFLD and alcohol whereas it is hepatitis B in Asia and Africa. Eighty percent occur in low and middle resource countries. Screening programme for HCC in cirrhotics is cost effective and better cancer surveillance can be achieved by the recently developed GALAD (incorporating AFP, AFP-L3, PIVKA, age and sex) screening tool[38] along with radiologic strategies (contrast enhanced ultrasonogram using Li-RADS, multiphasic CT/aMRI scan) every 6 mo in high risk patients. Ninety percent HCC occur on background CLD which pose additional health risk to HCC itself. Liver biopsy carries risk hence liquid biopsy using detection of circulating tumor cells specific to HCC, mutation or methylation of circulating tumor DNA, and transcriptomic profiling of extracellular vesicles are promising. The widely followed BCLC staging system have been upgraded (to be more inclusive for surgery with/without downgrading of tumor) and provide better platform for optimal use of different treatment modalities like ablation, resection, LT, stereotactic body radiation, locoregional and systemic therapy. LT has been extended outside Milan criteria to include more patients by various expanded selection criteria with reduced but excellent long term results[39]. Cancer in non cirrhotic liver is treated by LT with better understanding of transplant oncology. Newer drugs like multikinase inhibitors (Lenvatinib, Regorafinib, Cabozantinib, Ramucirumab), checkpoint inhibitors (atezolizumab, bevacizumab, durvalumab, pembrolizumab, nivolumab, ipilimumab and tremelimumab) are now available over sorafenib for systemic therapy with better outcome[40]. Limitations are their unclear safety profile in Child Pugh stage B, best response not more than 50%, unclear treatment sequence and use in early stage of tumor.

LT

ALD is now the predominant cause for LT followed by HCV and NAFLD in the West[41] whereas it is still HBV/HCV in the East with ALD at its heels[42]. With good patient selection, the present 1 year survival is 90% and 5 year of 70%. Good outcome has been substantially influenced by betterment of surgical techniques, perioperative management, organ preservation (normothermic machine perfusion), recipient selection (through organ sharing network), post transplant immunosuppressive management and of viral etiology (DAA for HCV. Bulevirtide/NA for HBV/HDV). The challenge of limited organ availability has been addressed by accepting marginal and extended criteria donors (donors of cardiac death, 30%-60% steatotic liver explant without inflammation, HBsAg and HCV positive donors), split liver grafts and live donor transplant especially in Asia[41]. Liver regeneration based approaches like stem cell therapy and organ bioengineering can also help. Most post transplant morbidity arise from prolonged use of immunosuppressive with resultant infections, hypertension, dyslipidemia, cardiovascular events, renal failure, malignancy and chronic organ rejection. Long term outcome can be improved by minimising/late introduction of standard IS or withdrawing it completely (20% become operationally tolerant) and using less toxic IS drugs (mToR inhibitors, interleukin 2 receptor blockers). With increased understanding of transplant immunology, research is on whether IS can be completely withdrawn after finite treatment by modulating recipient immunity (by CD4 Treg cells, regulatory dendritic cells or hematopoietic stem cell transplantation)[43]. The problem of HCV relapse leading to cirrhosis in 30% has been addressed by DAA. Present challenges for liver transplant are: (1) High alcohol recidivism; (2) ACLF grade 3 and severe acute alcohol related hepatitis; (3) NAFLD/non-alcoholic steatohepatitis with high comorbities; (4) Frailty in advance CLD; (5) Recipient and caregiver challenges; (6) Genetic variants; and (7) COVID-19. Severe acute respiratory syndrome coronavirus vaccine fails to decrease mortality as the patient's immunity is already weakened.

Artificial intelligence and digital transformation of various diagnostic modalities, decision making tool and management will further advance the treatment of liver diseases.

CONCLUSION

The current scenario of the epidemiology and the advances in diagnosis and treatment of liver diseases including their complications are summarised in this review.

FOOTNOTES

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MINIREVIEWS

Pancreatic acinar cell carcinoma: A comprehensive review

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Abstract

Acinar cell carcinoma (ACC) is a rare pancreatic malignancy with distinctive clinical, molecular, and morphological features. The long-term survival of ACC patients is substantially superior to that of pancreatic adenocarcinoma patients. As there are no significant patient series about ACCs, our understanding of this illness is mainly based on case reports and limited patient series. Surgical resection is the treatment of choice for patients with the disease restricted to one organ; however, with recent breakthroughs in precision medicine, medicines targeting the one-of-a-kind molecular profile of ACC are on the horizon. There are no standard treatment protocols available for people in which a total surgical resection to cure the condition is not possible. As a result of shared genetic alterations, ACCs are chemosensitive to agents with activity against pancreatic adenocarcinomas and colorectal carcinomas. The role of neoadjuvant or adjuvant chemoradiotherapy has not been established. This article aims to do a comprehensive literature study and present the most recent information on acinar cell cancer.

Key Words: Acinar cell carcinoma; Pancreas; Imaging; Immunohistochemical stains; Molecular features; Surgery; Chemotherapy

Core Tip: Acinar cell carcinoma (ACC) of the pancreas is a rare, malignant neoplasm that accounts for a small percentage of all pancreatic neoplasms. Our understanding of this disease remains unclear as there are no large series of patients with ACC. This review article aims to conduct a comprehensive literature review and present current knowledge about ACC.

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INTRODUCTION

The rising prevalence of abdominal imaging has resulted in an uptick in historically rare pancreatic cancer cases. The exocrine pancreas primarily comprises acinar and ductal cells[1]. Although acinar tissue accounts for most of the pancreatic organ, pancreatic neoplasms that mainly exhibit acinar differentiation are rare. The expression of various pancreatic enzymes, such as chymotrypsin, trypsin, and lipase, leads to acinar differentiation in the exocrine pancreas[1]. Most acinar neoplasms are solid, malignant, and not well-recognized[2]. Acinar cell cystadenoma is the sole benign acinar neoplasm documented; uncommon benign cystic, malignant cystic, and mixed carcinomas also exist.

Acinar cell carcinoma (ACC) of the pancreas is a rare, malignant tumor composed of cells with morphological resemblance to acinar cells and with evidence of exocrine enzyme synthesis by the neoplastic cells, accounting for 1%-2% of all pancreatic neoplasms[2-4]. ACC of the pancreas is a high-grade malignancy with no apparent site preference; it can occur in any section of the pancreas, but the head is the most common location.

Currently, there is no definitive course of treatment for ACC; however, vigorous surgical resection with negative margins has been linked to improved long-term survival[5]. Particularly with the use of computerized tomography (CT) and magnetic resonance imaging (MRI), as well as with the confirmation of fine needle aspiration (FNA) biopsies, early recognition and diagnosis are of particular relevance. Knowledge of these lesions' morphologic and immunohistochemical characteristics is also essential for a precise clinical diagnosis. In addition, extensive molecular analyses of acinar neoplasms have identified genomic alterations that may be amenable to specific therapies, thereby increasing the likelihood of widespread clinical molecular testing of these tumors.

This article aims to do a comprehensive literature study and present the most recent information on acinar cell cancer.

EPIDEMIOLOGY

ACC of the pancreas accounts for 1%-2% of all adult exocrine pancreatic neoplasms and 15% of pediatric neoplasms. ACC occurs most frequently in white males and is usually bimodal in age, with peaks in childhood at 8-15 years of age and adulthood with a peak incidence at 60 and is rare between the ages of 20 and 40. ACC has a 3.6 male-to-female ratio. No known racial relationships exist[1,6,7].

CLINICAL FEATURES

Most ACC s exhibit vague symptoms, which include weight loss (45%), abdominal pain (60%), back pain (50%), nausea and vomiting (20%), melena (12%), weakness, anorexia, and diarrhea (8%) are typical non-specific symptoms of ACC[8,9]. In distinction to ductal adenocarcinoma, ACC infrequently obstructs the bile duct and causes jaundice only in 12% of patients. ACC causes a mass effect and displaces those organs but does not infiltrate[10].

Some patients experience paraneoplastic disease known as "Schmid's Triad" or lipase hypersecretion syndrome due to increased lipase levels reaching over 10000 U/dL[11,12]. Elevated lipase may be the first sign of ACC. Patients may present with multiple nodular foci of subcutaneous fat necrosis, polyarthralgia may occur due to fat necrosis in the cancellous bone, and may have peripheral blood eosinophilia[13,14]. On imaging, fat necrosis within the bone can appear as a focal area of sclerosis.

Paraneoplastic syndrome may occur following tumor recurrence. Most patients have hepatic metastases at presentation; however, some may present with only a bulky pancreatic mass. The serum lipase levels may normalize after surgical resection of the tumor, and patients may have symptomatic relief. Lipase may be used as a tumor marker in these patients. Alpha-fetoprotein (AFP) blood levels can be elevated in young patients[15].

MACROSCOPY (GROSS FEATURES)

On FNA, an abundance of cells with fluctuating degrees of acinar differentiation but no endocrine or ductal cells are seen. The aspirates consist primarily of cohesive fragments creating acini, cellular cords, or solid nests of neoplastic epithelium, composed of clusters of uniform cells with smoothly contoured, eccentric nuclei containing one or two prominent nuclei nucleoli[16,17].

ACCs can occur in any region of the pancreas and, on average, are 10 to 11 cm in size at the time of diagnosis[18-20]. The tumors are well circumscribed and have a soft, fleshy, tan to red appearance[2]. They occasionally exhibit lobulated appearances, necrosis, and cystic degeneration[20]. On occasion, the neoplasm is found attached to the surface of the pancreas. Involvement of the duct system by ACCs can sometimes be observed grossly as polypoid or fingerlike projections extending from the primary tumor mass toward the duct[21].

PATHOLOGY

ACCs are cellular neoplasms with minimal stroma on microscopic examination. Multiple architectural patterns, including acinar (Figure 1A), solid (Figure 1B), glandular (Figure 1C), and trabecular (Figure 1D), can be observed. Solid and acinar patterns are the most prevalent. The acinar pattern is characterized by structures resembling normal acini, with small lumina and cells arranged in a monolayer with basally located nuclei. Large sheets of cells depict the solid pattern without lumina. Acinar structures with the dilated lumen characterize the less common granular pattern. The trabecular pattern, which resembles the architecture of well-differentiated neuroendocrine tumors, consists of more pronounced palisading with thinner, interlacing ribbons of cells. Multiple architectural patterns are visible within a single tumor.

IMMUNOHISTOCHEMISTRY

The immunohistochemical labeling of enzyme production from pancreatic cells aids in confirming the diagnosis of ACC (Table 1). There are commercially available antibodies for chymotrypsin, trypsin, lipase, and amylase, with the first three being the most frequently used in clinical settings (Figure 1E). These enzymes exhibit varying degrees of specificity and sensitivity. In approximately 95% of cases, trypsin and chymotrypsin reactivity can be demonstrated, and this combination is reportedly the most sensitive for diagnosing ACC[10,22].

ACC is also positive for CK8 and CK18, and as a result, they will label for AE1/AE3 and CAM5.2. CK7 (Figure 1F) and CK19, which are usually expressed in ductal adenocarcinomas, are generally missing in ACC but may be expressed in a subgroup of ACCs[15,23]. Focal labeling for chromogranin and synaptophysin is observed in a substantial proportion of ACCs, displaying the need to assess with further clear-cut acinar markers, especially in small samples, to avoid misidentifying an ACC as a neuroendocrine tumor or mixed acinar-neuroendocrine differentiation[10,24,25]. Recent research on histological and cytological samples has proven that the monoclonal antibody directed against the COOH-terminal region of the BCL10 protein, which identifies the COOH-terminal portion of CEL, is a highly specific and sensitive approach for detecting ACCs[26,27].

MOLECULAR FEATURES

The genetic alterations driving ACC have been thoroughly characterized and sequenced. The outcomes of these studies have emphasized the diversity of modified genes and pathways among various tumors, as well as the marked genomic instability in a subset of instances, including at the base pairs and chromosomes level. Several studies analyzed ACC for mutations in gene changed in pancreatic ductal adenocarcinoma (PDAC), the most prevalent subtype of pancreatic cancer, before the wide availability of whole genome and exome. Several of these studies reported uncommon mutations in *TP53*, *KRAS*, and *SMAD4*, in addition to the unusual loss of SMAD4 expression in acinar neoplasms, while others reported no modifications in these genes[28-33]. In these studies, the most prevalent ACC mutations

Table 1 Immunohistochemical profile from the differential diagnosis

Tumor type	PCK	Trypsin/chymotrypsin/BCL-10	BCL-10	B-Catenin	Synaptophysin	Chromogranin	Others
ACC	+	+	+	+/-	-/+	-/+	Amylase. Lipase. PAS
PDAC	+	-	-	-	-	-	P53, DPC4 loss
SPN	-/+	-	-	+[1]	-	-	PR, CD56, Alpha 1-antitrypsin
PanNET	+	-	-	-	+	+	CD56, PAX8[3], ISL1[3]
PBL	+	+	-	+[2]	-	-	Alpha fetoprotein, CEA

[1]Nuclear.
[2]Nuclear/Cytoplasmic and positive in squamous morules.
[3]PAX8 and ISL1 are specific markers for PanNETs.
ACC: Acinar cell carcinoma; PCK: Pancytokeratin; PanNET: Pancreatic neuroendocrine tumors; PBL: Pancreatoblastoma; SPN: Solid pseudopapillary neoplasm; PDAC: Pancreatic ductal carcinoma; PAS: Periodic acid-Schiff; ISL 1: Islet 1; CEA: Carcinoembryonic antigen.

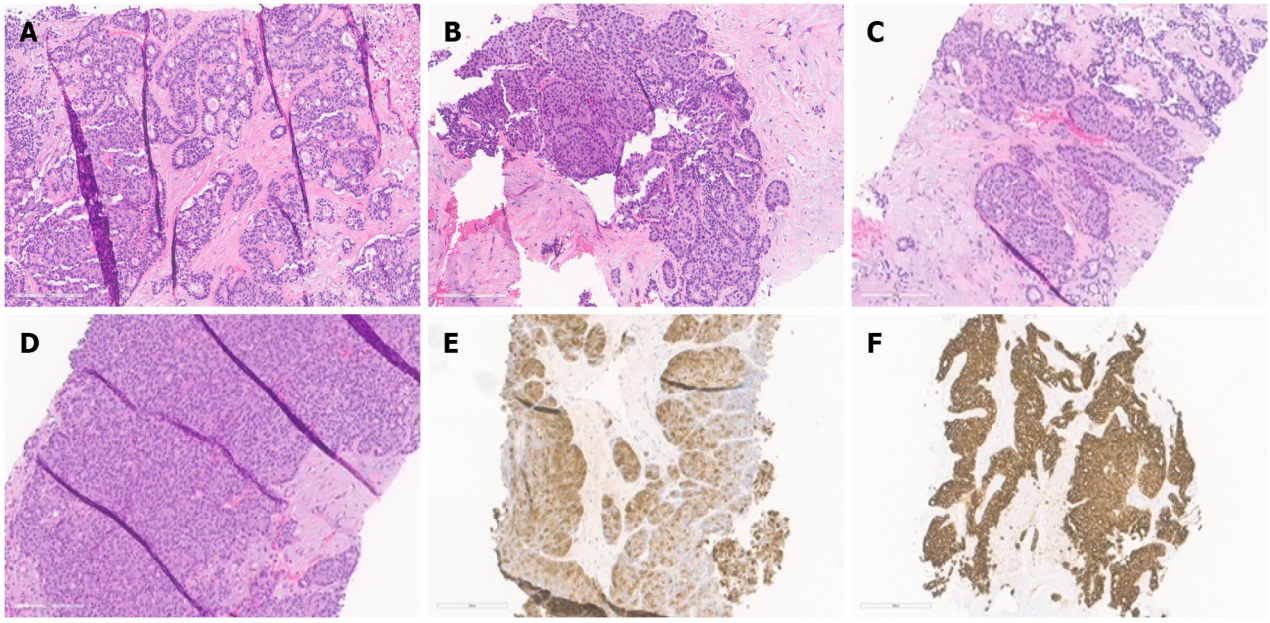

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Figure 1 Histopathological patterns. A: Acinar cell carcinoma with the predominant acinar pattern. The acinar pattern is characterized by structures resembling normal acini, with small lumina and cells arranged in a monolayer with basally located nuclei; B: Acinar cell carcinoma with the predominant glandular pattern. Acinar structures with dilated lumina characterize the glandular pattern; C: Acinar cell carcinoma with the predominant trabecular pattern. The trabecular pattern is characterized by ribbons of cells resembling those of pancreatic neuroendocrine tumors; D: Acinar cell carcinoma with a predominant solid pattern. Large sheets of cells characterize the solid pattern without lumina; E: Acinar cell carcinoma, immunohistochemical staining for trypsin; F: Acinar cell carcinoma, immunohistochemical staining for cytokeratin 7.

affected WNT signalings, such as mutations in APC that rendered inactive and mutations in CTNNB1 that rendered it active; these alterations were found in roughly 20% of ACCs[30]. Recent sequencing studies of the entire exome have highlighted the diversity of mutated genes in the ACC[34,35]. Several other studies have shown substantial chromosomal gains and losses in ACCs and altered regions in various carcinomas; nevertheless, the target genes (if any) in these regions have not been detected[29,30,36,37].

Multiple potentially targetable genetic alterations in pancreatic ACC have been identified. A subset of cases exhibits genomic instability due to mutations in genes involved in DNA repairs, such as *ATM*, *BRCA1*, *BRCA2*, *PALB2*, and *MSH2*[34,35]. According to various studies, microsatellite instability has been documented in fluctuating proportions of ACC, ranging from 7% to 14%[30,35,38]. Also, platinum agents and PARP inhibitors treat tumors with mutations in BRCA pathway genes[39,40]. Even though it has not yet been proven in major clinical trials, preliminary findings indicate that tumors with BRAF fusions react to MEK inhibitors, providing ACC with targeted therapy[41-43]. In a subgroup of ACCs,

Table 2 Summary of pertinent differential diagnostic features

Differential diagnoses	Age/sex	Imaging/gross findings	Histology	Prognosis
ACC	Predominant in males; mean age of 62 yr	Solid, well-circumscribed, bulky tumors; hemorrhage and necrosis are also frequent	Predominant acinar or solid architecture; uniform cells; basally located nuclei; eosinophilic granular cytoplasm; prominent single nucleoli. minimal stroma	Overall aggressive, with high rates of recurrence and metastasis
PDAC	Slightly higher in males; 6th-8th decade of life	Solid, poorly defined mass	Large, medium, or small malignant ducts with a tubular pattern; desmoplastic stroma. Processes of mitosis and necrosis	Poor survival rates
PanNET	Even distribution between the genders; more prevalent in adults; mean age of 40	Solid, well-circumscribed. 5% are cystic	Variable architectural patterns; uniform cells; oval or spherical nuclei; granular cytoplasm; undetected nucleoli; minimal stroma	Relatively languid, but with variable results
SPN	Almost exclusively female; average age of 28	Well-defined and encased with cystic degeneration	Pseudopapillae; cells with hyaline/myxoid stroma surrounding vessels; large cytoplasmic hyaline globules; nuclear groove	Overall low malignant potential: The majority are successfully treated surgically
PBL	First decade of life, mean age of 4; adults can be affected	Partially encapsulated, frequently lobulated, and substantial	Solid and acinar structure; cellular stroma; keratinization of squamoid nests; heterologous mesenchymal elements	Aggressive; better outcomes for children

ACC: Acinar cell carcinoma; PanNET: Pancreatic neuroendocrine tumors; PBL: Pancreatoblastoma; SPN: Solid pseudopapillary neoplasm; PDAC: Pancreatic ductal carcinoma

MYC abnormalities, including chromosome 8 polysomy and gene amplification, have been identified recently[44].

DIAGNOSIS

Laboratory analysis

Besides an increase in serum lipase levels associated with the syndrome of lipase hypersecretion, serum tumor markers are not consistently elevated in ACC. Even in the absence of lipase hypersecretion syndrome, lipase might be high. There have been numerous AFP increases, especially in younger patients[2]. Typically, Serum tumor markers such as carbohydrate antigen 19-9, alpha-fetoprotein, and carcinoembryonic antigen are expressed.

Radiological features (imaging)

In recent years, the incidence of uncommon pancreatic cancers has grown, likely due to the increased use of cross-sectional imaging[45]. In most instances, pancreatic cancers are incidentally found by CT. MRI is often used as a secondary test for further characterization. ACC of the pancreas is a well-defined, primarily oval or round exophytic mass in cross-sectional imaging[46,47]. Rarely have cystic variations been described. Typically, it manifests as a dense, largely solid tumor devoid of noticeable cystic alterations[46,48]. One-third of patients may exhibit calcifications, and the majority of the tumor increases constantly, but less so than the adjacent pancreatic parenchyma[49]. The tumor displays heterogeneous enhancement after intravenous infusion of contrast material[46]. Other studies also concluded that when confronted with a large mass with an ovoid shape, exophytic, well-circumscribed arising from the pancreas, with slight and persistent enhancement after contrast administration, and without appreciable biliary or pancreatic dilation, the diagnosis of ACC should be considered[18,47,50]. Another study stated that MRI is better than CT for identifying tumor margination, intratumoral bleeding, tissue invasion, and ductal dilation[51]. On the other hand, CT is more sensitive to detecting central calcification and is commonly utilized due to its accessibility and quickness[50,51]. CT and MRI accurately depict most ACC imaging characteristics, and the two modalities correlate well[51].

Differential diagnosis

Pancreatic endocrine neoplasms that are well-differentiated, solid pseudopapillary neoplasms (SPNs), mixed acinar neoplasms, pancreatoblastoma (PBL), and PDACs should be included in the differential diagnosis (Table 2). Well-differentiated endocrine neoplasm, also commonly known as pancreatic neuroendocrine tumors (PanNET), is the entity most widely confused and misdiagnosed[2]. Both neoplasms typically contain a high number of cells and little amount of stroma, are capable of forming cell sheets and can consist of medium-sized, spherical, monomorphic cells with a microglandular or

solid pattern of growth[2]. In addition, the red cytoplasmic zymogen and neurosecretory granules from ACC and PanNETs, respectively, are similar with appropriate differentiation. PanNETs are associated with a more favorable prognosis, making differentiation crucial. ACCs are distinguished from PanNETs by their primary acinar development, cytoplasmic granulation, basally oriented nuclei, and individual conspicuous nucleoli. However, hyalinized stroma, plasmacytoid cell appearance, and a "salt and pepper" chromatin pattern are indicative of PanNET[6]. Immunohistochemistry is advantageous, but it must be utilized with care. Using general neuroendocrine markers such as synaptophysin and chromogranin A alone may be unsafe because several ACCs contain neuroendocrine cells, which are particularly abundant in 30% of cases, as stated before[10,24]. Therefore, the demonstration of acinar cell products is mandatory if an ACC is suspected.

SPNs present as massive, dense pancreatic masses that are clinically identical to ACC; however, SPNs virtually entirely affect women of young age[52]. Cytological smears are characterized by delicately branched vasculature with lightly adherent cells generating pseudopapillary structures, a homogenous population of microscopic cells with cuboidal formation, and a unique hyaline myxoid matrix[52]. Additionally, cytoplasmic hyaline globules are occasionally visible. Moreover, the SPNs nuclei are often normochromic, with numerous indentations, and lack the conspicuous nucleoli found in acinar cells [53]. Typically, SPNs exhibit nuclear immunoreactivity for β-catenin and high expression of CD10, which are also detectable in roughly 10% and 60% of ACCs, respectively. As a result, these two markers must be utilized in conjunction with markers specific to acinar cells, such as trypsin and BCL10[53-55].

PBL is another entity with stroma-poor acinar differentiation that must be evaluated in the differential diagnosis. Similar to ACCs, upon the appearance, PBLs are typically large, with an average size of 10 cm ranging from 1.5 to 20 cm[56]. This uncommon tumor shares nearly all of the cytologic and clinical characteristics of ACC, notably in the population of younger age, where it is frequently observed [2,57]. Cytologically, the neoplasms may be indistinguishable because they both display a predominant acinar-cell differentiation[2]. Heterologous components and squamous nests, such as osseous and cartilage development, characterize PBL[2]. In addition to immunochemistry and clinicopathologic data, the heterogeneous cytologic elements of the types of the tumor-cell present, including a combination of squamoid, mesenchymal, and glandular cells, are a general clue to diagnosing PBL[58]. Histologically, the presence of squamoid nests in PBL is the distinguishing characteristic of the two entities.

ACCs typically lack the characteristic cytomorphologic features of PDACs, allowing these two tumors to be distinguished easily. PDACs typically exhibit "drunk honeycomb" and "tombstone cells" arrangements alongside nuclear heterogeneity and nuclear silhouette abnormalities[59]. This is in contrast to the relatively homogeneous polygonal cells of ACCs, which are arranged in three-dimensional arrays and tissue fragments[1]. ACCs lack the large desmoplastic stroma that describes PDACs and display an overall solid and acinar architecture, whereas PDACs feature a prominent desmoplastic stroma[10,59,60].

STAGING FOR ACINAR PANCREATIC CARCINOMA

The staging system for acinar pancreatic cancer is undergoing continuous development. Clinical staging is determined by resectability, which is heavily impacted by surgical expertise. The agreed guidelines for the resectability of surgical procedures continue to be refined (*e.g.*, National Comprehensive Cancer Network, MD Anderson Cancer Center, American Hepato-Pancreato-Biliary Association, and International Hepato-Pancreato-Biliary Association). Traditionally, they are categorized as resectable, borderline resectable, locally progressed, and metastatic. To begin with, resectable tumors lack vascular involvement. Next, the borderline resectable tumors involve vascular, local structures, or other signs indicating a high risk of R1 resection. Local invasions (particularly vascular involvement) prevent surgical intervention for locally progressed malignancies. Lastly, metastatic cancer has spread to other organs beyond the main pancreatic tumor. The American Joint Committee on Cancer (AJCC) has classified staging by TNM (tumor, node, metastasis) classification using the consensus guidelines for resectability (Table 3 and Figure 2)[61]. Imaging cases with different AJCC TNM staging were added in chronological order (Figures 3 - 7) to appreciate the differences in changes.

PROGNOSIS, FACTORS OF PREDICTION, SPREAD, AND METASTASES

ACCs are exceptionally malignant neoplasms; relatively 50% of patients have metastasis during diagnosis[62]. Initial studies indicate that the prognosis for ACCs is better than that for ductal carcinomas but worse than that for PanNETs[5,63,64]. Previous research using population-based cancer databases and epidemiological methods indicated that individuals diagnosed with ACCs have a median overall survival time of roughly 47 mo for patients with localized disease and 14 mo for patients with metastatic disease, with a 5-year survival rate ranging from 36.2% to 72.8% for resected cancers[5,64,65]. A recent study shows that ACC has a better prognosis than adenocarcinoma. They found that the prognosis was deteriorating with a decrease of 25% in a 5-year survival rate to previously reported rates

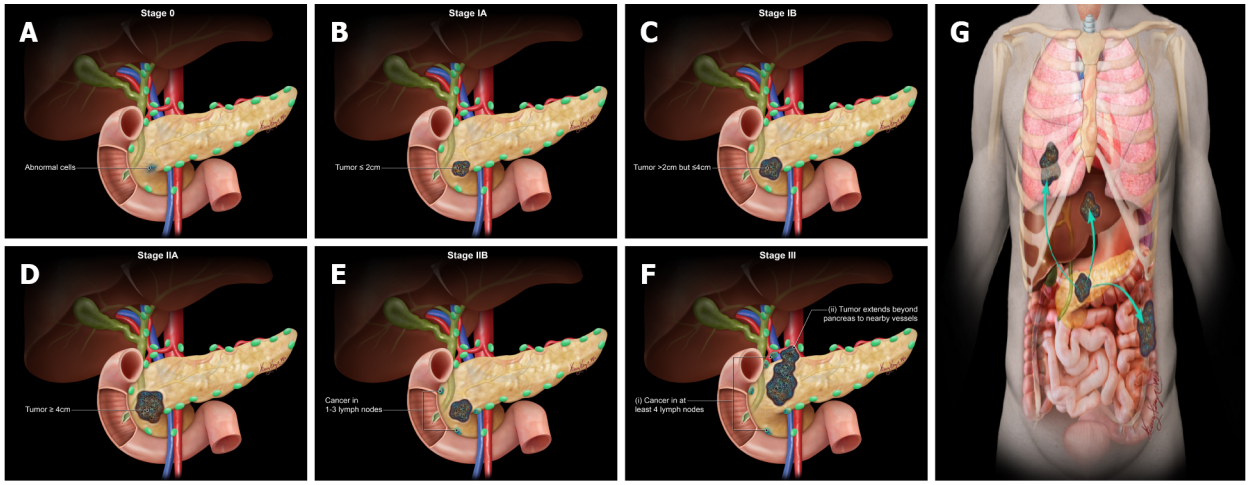

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Figure 2 American Joint Committee on Cancer 8th Edition cancer staging for exocrine pancreatic tumor such as pancreatic acinar cell carcinoma. A: Stage 0 (TNM: Tis, N0, M0). Tis = Carcinoma in situ. N0 = No regional lymph node metastases. M0 = No distant metastasis; B: Stage IA (TNM: T1, N0, M0). T1 = Tumor ≤ 2 cm in greatest dimension. N0 = No regional lymph node metastases. M0 = No distant metastasis; C: Stage IB (TNM: T2, N0, M0). T2 = Tumor > 2 cm and ≤ 4 cm in greatest dimension. N0 = No regional lymph node metastases. M0 = No distant metastasis; D: Stage IIA (TNM: T3, N0, M0). T3 = Tumor > 4 cm in greatest dimension. N0 = No regional lymph node metastases. M0 = No distant metastasis; E: Stage IIB (TNM: T 1/2/3, N1, M0). T 1/2/3 = Tumor ≤ 2 cm > 4 cm in greatest dimension. N1 = Metastasis in one to three regional lymph nodes. M0 = No distant metastasis; F: Stage III (TNM: T 1/2/3, N2, M0 or T4, Any N, M0). T 1/2/3 = Tumor ≤ 2 cm > 4 cm in greatest dimension. N2 = Metastasis in four or more regional lymph nodes. T4 = Tumor involves celiac axis, superior mesenteric artery, and common hepatic artery, regardless of size; G: Stage IV (TNM: Any T, Any N, M1). M1 = Distant metastasis. T = Primary tumor; N = Regional lymph node; M = Distant metastasis.

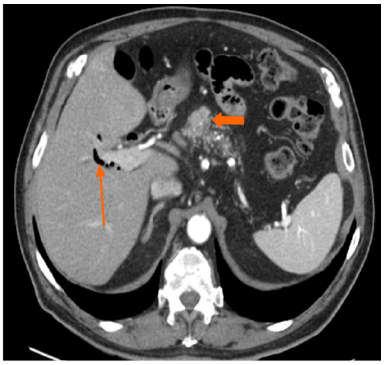

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Figure 3 Stage IA (T1, N0, M0). A 77-year-old male patient with acinar cell carcinoma. Axial post-contrast portal-venous phase computed tomography image shows a solid mass (short arrows) measuring 1.8 cm × 1.6 cm and involving the region of the pancreatic head/body. Incidental findings are calcifications seen throughout the pancreas (likely related to changes in chronic pancreatitis) and mild dilatation of the biliary tree with pneumobilia (long arrow).

[66]. However, data from a few studies published and anecdotal reports hint that some patients with ACCs have a greater survival rate than expected; unfortunately, no parameters are available to identify this group of patients[67,68].

Rarely do patients with ACC are diagnose with lung, cervical lymph nodes, and ovary metastases. Metastatic illness is typically detected in regional lymph nodes and the liver[69]. Patients presenting as their first disease symptom with distant metastases are infrequent. It has been proposed that ACCs in patients younger than 20 may be less aggressive than in adults[10]. The most significant prognostic indicator is the tumor stage, with patients without lymph nodes or distant metastases having a better prognosis[5,70]. In addition, a poor prognosis is also associated with being male, being over 60, and having a tumor larger than 10 cm[5].

TREATMENT

Current, surgical, radiotherapeutic, and chemotherapeutic treatments used for PDAC are typically used to treat ACC (Table 4). Surgery is regarded as the optimal treatment modality for ACCs that are

Table 3 American Joint Committee on Cancer 8th Edition cancer staging for exocrine pancreatic tumor such as pancreatic acinar cell carcinoma

Stage	TNM	Description
0	Tis	Carcinoma in situ[1]
	N0	No regional lymph node metastases
	M0	No distant metastasis
IA	T1	T1 = Tumor is ≤ 2 cm in any direction
		T1a = Tumor is ≤ 0.5 cm in any direction
		T1b = Tumor is > 0.5 cm and < 1 cm in any direction
		T1c = Tumor is 1–2 cm in any direction
	N0	No regional lymph node metastases
	M0	No distant metastasis
IB	T2	Tumor is > 2 cm and ≤ 4 cm in any direction
	N0	No regional lymph node metastases
	M0	No distant metastasis
IIA	T3	Tumor is > 4 cm in any direction
	N0	No regional lymph node metastases
	M0	No distant metastasis
IIB	T1	T1 = Tumor is ≤ 2 cm in any direction
		T1a = Tumor is ≤ 0.5 cm in any direction
		T1b = Tumor is > 0.5 cm and < 1 cm in any direction
		T1c = Tumor is 1–2 cm in any direction
	N1	Metastasis in one to three regional lymph nodes
	M0	No distant metastasis
	T2	Tumor is > 2 cm and ≤ 4 cm in any direction
	N1	Metastasis in one to three regional lymph nodes
	M0	No distant metastasis
	T3	Tumor is > 4 cm in any direction
	N1	Metastasis in one to three regional lymph nodes
	M0	No distant metastasis
III	T1	T1 = Tumor is ≤ 2 cm in any direction
		T1a = Tumor is ≤ 0.5 cm in any direction
		T1b = Tumor is > 0.5 cm and < 1 cm in any direction
		T1c = Tumor is 1–2 cm in any direction
	N2	Metastasis in four or more regional lymph nodes
	M0	No distant metastasis
	T2	Tumor is > 2 cm and ≤ 4 cm in any direction
	N2	Metastasis in four or more regional lymph nodes
	M0	No distant metastasis
	T3	Tumor is > 4 cm in any direction
	N2	Metastasis in four or more regional lymph nodes
	M0	No distant metastasis
	T4	Regardless of tumor size, the cancer has grown outside the pancreas, into the nearby

		large blood vessels[2]
	Any N	NX = Regional lymph nodes cannot be assessed
		N0 = No regional lymph node metastases
		N1 = Metastasis in one to three regional lymph nodes
		N2 = Metastasis in four or more regional lymph nodes
	M0	No distant metastasis
IV	Any T	TX = Primary tumor cannot be assessed
		T0 = No evidence of primary tumor
		Tis = Carcinoma in situ[1]
		T1 = Tumor is ≤ 2 cm in any direction
		T1a = Tumor is ≤ 0.5 cm in any direction
		T1b = Tumor is > 0.5 cm and < 1 cm in any direction
		T1c = Tumor is 1–2 cm in any direction
		T2 = Tumor is > 2 cm and ≤ 4 cm in any direction
		T3 = Tumor is > 4 cm in any direction
		T4 = Regardless of tumor size, the cancer has grown outside the pancreas, into the nearby large blood vessels[2]
	Any N	NX = Regional lymph nodes cannot be assessed
		N0 = No regional lymph node metastases
		N1 = Metastasis in one to three regional lymph nodes
		N2 = Metastasis in four or more regional lymph nodes
	M1	Distant metastasis

[1]Included in this category are high-grade pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplasm with high-grade dysplasia, intraductal tubulopapillary neoplasm with high-grade dysplasia, and mucinous cystic neoplasm with high-grade dysplasia.
[2]Celiac axis, superior mesenteric artery, and/or common hepatic artery are involved.
T: Primary tumor; N: Regional lymph node; M: Distant metastasis.

Table 4 Acinar cell carcinoma treatment options

Clinical stage	Treatment options
Resectable or borderline resectable	Neoadjuvant therapy[1]
	Surgery
	Postoperative chemotherapy
	Postoperative chemoradiation therapy
Locally advance	Chemotherapy[2]
	Chemoradiation therapy
	Surgery
	Palliative surgery
Recurrent or metastatic	Chemotherapy[2]

[1]Chemotherapy with or without chemoradiation therapy given before surgery.
[2]With or without target therapy.

regionally circumscribed and resectable. Holen *et al*[8] found that median survival for surgically resected cases was 36 mo, compared to 14 mo for those without surgical resection[8]. Another systematic research by Glazer *et al*[71] indicated that the total median survival rate for patients with ACC who had resection was approximately 47 mo[71]. More aggressive therapeutic regimens must be

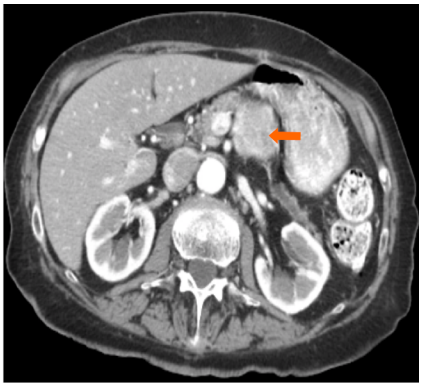

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Figure 4 Stage IB (T2, N0, M0). An 89-year-old male patient with acinar cell carcinoma. Axial post-contrast computed tomography arterial phase image shows a large mass in the body of the pancreas (arrow) measuring 3.3 cm × 3 cm. No regional adenopathy is identified.

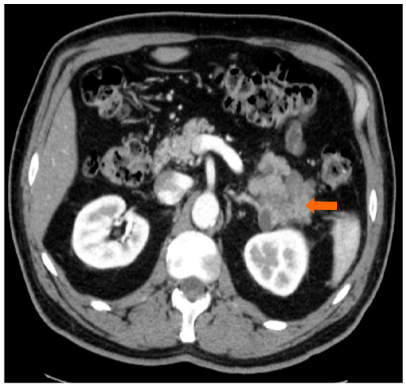

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Figure 5 Stage IIA (T3, N0, M0). A 66-year-old patient with acinar cell carcinoma. Axial post-contrast computed tomography image in the arterial phase shows a solid lobulated mass measuring 6.5 cm × 5.4 cm arising from the tail of the pancreas (arrow). The mass is heterogeneous in density with areas of low-density and solid-enhancing areas. No regional or distant lymphadenopathy was detected.

sought when surgery is unavailable for locally advanced and metastatic diseases.

Due to the rareness of ACC and the absence of sufficient randomized trials comparing different treatment approaches, the role of adjuvant therapy remains debatable. Table 5 enlists characteristics of studies on various regimes used for treating ACC. In a study at Memorial Sloan-Kettering Cancer Center, systemic chemotherapy for ACC was determined to be ineffective[8]. Following surgical resection, there are no clear treatment guidelines, and most adjuvant therapies are individualized with variable response rates. Lack of prospective data to create therapy guidelines and genetic variation in the *APC* gene/β-catenin pathway observed in pancreatic acinar cells, ACC patients are frequently treated with chemotherapeutic agents known to be active against PDAC or colorectal cancer[72]. Most study participants received combination chemotherapy protocols based on gemcitabine or fluoropyrimidine[11,73]. If the ACC cannot be resected, the patient should undergo neoadjuvant or palliative 5-FU chemotherapy[70,74]. Combination fluoropyrimidine-based chemotherapies have increased disease control rates[8,11,73,75-77]. Glazer *et al*[71] systematic review showed that patients with a high-performance status typically receive folinic acid/fluorouracil/oxaliplatin or folinic acid/fluorouracil/irinotecan. In contrast, patients with a lower performance status typically receive gemcitabine/protein-bound paclitaxel[71].

Conventional fractionation and hypofractionated stereotactic body radiotherapy are used to "down-stage" or convert a borderline resectable tumor to one that is resectable[73]. Patients who could potentially benefit from resection can be identified through the absence of ACC development following neoadjuvant radiotherapy. Several studies reported a "major response" rate from 25% to 35% of these individuals[8,11].

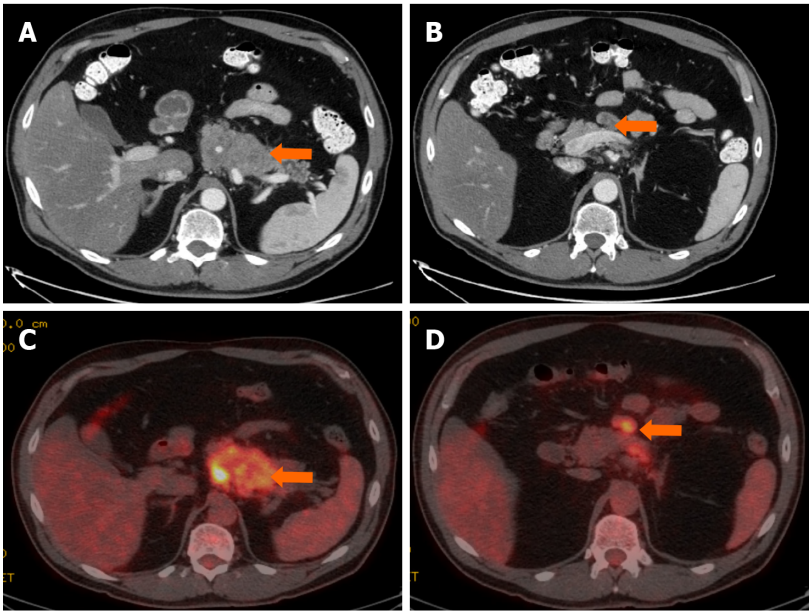

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Figure 6 Stage IIB (T3, N1, M0). A 61-year-old patient with acinar cell carcinoma. A: Axial post-contrast computed tomography (CT) image in the portovenous phase shows an infiltrative mass arising from the pancreatic body and tail (arrow); B: axial post-contrast CT images in the portovenous phase shows an enlarged mesenteric lymph node (arrow) measuring 2.6 cm × 1.2 cm; C: Axial positron emission tomography/CT image in the portovenous phase shows hypermetabolic pancreatic body and tail mass (arrow); D: Axial post-contrast CT images in the portovenous phase shows hypermetabolic enlarged mesenteric lymph node (arrow). Pathology of the mass revealed Acinar cell carcinoma with a metastatic mesenteric lymph node.

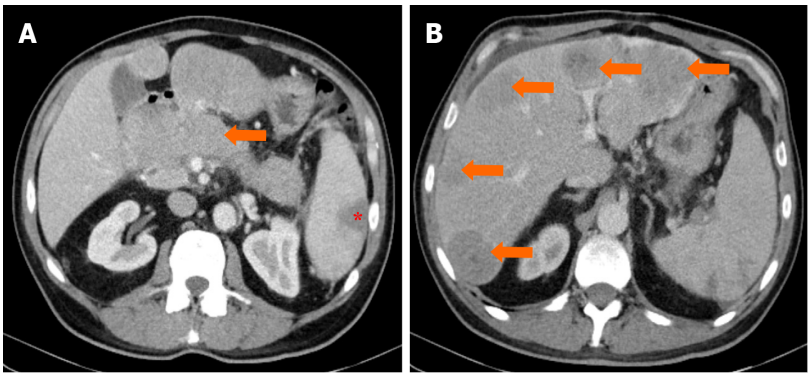

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Figure 7 Stage IV. A 64-year-old patient with acinar cell carcinoma. A: Axial post-contrast image port venous phase shows a pancreatic head mass measuring about 3.2 cm × 2.8 cm (arrow); B: Axial post-contrast image port venous phase shows multiple bilobar variable-sized hepatic metastatic lesions (arrows). An incidental finding is an area of splenic infarction (asterisk in image A).

CONCLUSION

Pancreatic acinar neoplasms are a molecularly and morphologically heterogeneous group of diseases far less prevalent than ductal neoplasms. Due to its low surgical resectability, high rate of recurrence, and high frequency of metastases at diagnosis, pancreatic ACC has been regarded as cancer with a poor prognosis. At least a subset of the neoplastic cells exhibits acinar differentiation, as defined morphologically (granular cytoplasm, single prominent nucleoli) or immunohistochemically. The molecular system underlying the advancement of ACCs has been elucidated, revealing potentially targetable mutations that offer optimism for developing additional curative options. ACC usually appears as a bulky mass that does not obstruct the pancreatic or bile duct and does not cause jaundice. Elevated lipase levels, subcutaneous nodules, and the presence of bone infarct should help clinch a diagnosis of ACC. Also, these tumors have large lymph nodes at presentation. In the future, molecular diagnostics to detect lesions susceptible to targeted therapy will likely play an important role in patient care.

Table 5 Characteristics of studies on various acinar cell carcinoma treatment regimes

Ref.	Year of publication	ACC sample size	Type of treatment	No. of patients	Conclusion
Holen *et al* [8]	2002	39	Resection	9	A high recurrence rate following complete surgical resection suggests that micrometastases are present even in localized disease, and that adjuvant therapies may be indicated. Chemotherapy and radiation are ineffective, however, and novel treatments are required
			RT alone	22	
			Fluoropyrimidine-based chemotherapy and RT	1	
			Fluoropyrimidine-based chemotherapy	7	
Kitagami *et al*[65]	2007	115	Resection	88	To improve prognosis, surgical resection should be pursued if possible. If ACC cannot be resected or recurs, chemotherapy is likely to be beneficial. A multidisciplinary treatment centered on the role of surgery must be developed
			Palliative operation	12	
			Exploratory laparotomy	4	
			Other treatment[1]	11	
Seth *et al* [11]	2008	14	Resection	10	When feasible, surgical resection is the optimal first-line treatment for resectable ACC due to its superior survival, which can be further improved by the addition of a planned neoadjuvant and/or adjuvant chemoradiation regimen
			Resection, mixed chemotherapy[2] and RT	4	
Wisnoski *et al*[64]	2008	672	Resection	266	ACC surgical resection appears to improve survival, and the findings support an aggressive strategy for resectable disease. In order to define the role of chemoradiation in the palliative, adjuvant, and neoadjuvant settings, additional research is required
			Other treatment[1]	406	
Schmidt *et al*[5]	2008	865	Resection	190	In these favorable pancreatic cancers, aggressive surgical resection with negative margins is associated with long-term survival. Second, cancer registries lack certain information, such as the specific type of chemotherapy administered and radiation therapy details. Consequently, institutional and multi-institutional reports of ACC continue to be essential for performing a more comprehensive analysis of the presentation, pathology, natural history, and treatment-related outcomes of ACC
			Resection and chemotherapy	33	
			Resection and RT	10	
			Resection and chemoradiation	100	
			Other treatment[1]	532	
Matos *et al* [75]	2009	17	Resection	12	ACC requires aggressive surgical resection. Importantly, some patients with locally advanced ACC have responded to a neoadjuvant approach allowing resection of a downstaged tumor; therefore, a combined modality approach should be considered for these patients
			Mixed chemotherapy[2]	3	
			Mixed chemotherapy[2] and RT	2	
Seki *et al* [76]	2009	4	Gemcitabine-based chemotherapy	1	A partial response suggested that fluoropyrimidine-based chemotherapy may have some activity against this tumor. To confirm the efficacy of fluoropyrimidine in treating pancreatic ACC, prospective clinical trials are required
			Fluoropyrimidine-/gemcitabine-based chemotherapy	3	
Lee *et al* [78]	2010	29	Resection	12	In Korea, the clinical characteristics of ACC include a young age, a large size, a location in the tail, and nonspecific tumour markers. ACC should always be actively treated with surgery, regardless of its size
			Resection, mixed chemotherapy[2] and RT	10	
			Mixed chemotherapy[2] and RT	1	
			Other treatment[1]	6	
Butturini *et al*[73]	2011	9	Resection	2	Using multiple chemotherapy regimens and regional treatments sequentially for recurrent disease allowed for 45-, 85-, and 52-mo post-primary survival. Long-term survival and clinical benefit may be possible with repeated surgery, neoadjuvant and adjuvant chemoradiation therapies, and locoregional therapy
			Resection and gemcitabine-based chemotherapy	7	
Hartwig *et al*[79]	2011	17	Resection	13	ACC of the pancreas is a relatively uncommon tumor entity for which resection may lead to long-term survival, even in the presence of limited metastatic disease. Optimized adjuvant treatment protocols are required to improve the long-term survival of ACC patients
			Resection and gemcitabine-based chemotherapy	4	

Lowery *et al*[77]	2011	20	Gemcitabine-based chemotherapy	20	Observed efficacy of combination chemotherapy in metastatic patients. ACC supports the use of combination therapies based on gemcitabine or 5-fluorouracil and incorporating irinotecan, a platinum analog, or docetaxel in patients with advanced disease. A potential association between germline mutations in DNA mismatch repair genes and ACC warrants further evaluation
Zheng *et al*[80]	2015	15	Resection	12	Clinicians generally regard pancreatic acinar cell carcinoma as a low-grade malignancy due to its unique clinical features. Positive sentiments towards ACC should be held
			Resection and gemcitabine-based chemotherapy	3	
Kruger *et al*[81]	2016	15	Resection	3	In contrast to PDAC, gemcitabine alone does not appear to have significant activity in ACC. Based on the findings, advanced ACC should be treated with chemotherapy regimens containing 5-FU and/or a platinum compound (such as oxaliplatin). Undetermined is whether this observation also applies to adjuvant chemotherapy administered after surgical resection of ACC
			Resection and gemcitabine-based chemotherapy	8	
			Chemoradiation	1	
			Mixed chemotherapy[2]	3	
Seo *et al* [82]	2017	20	Resection	9	Compared to PDAC, patients with resectable pancreatic ACC had a favorable prognosis after curative resection. Although adjuvant chemotherapy was not associated with improved survival in this study, it is unknown whether this was due to a selection bias or the ineffectiveness of 5-FU monotherapy in pancreatic ACC. On the basis of molecular analysis utilizing innovative genetic analytic tools, additional research on effective adjuvant chemotherapy is required
			Resection and 5-fluorouracil-based chemotherapy	9	
			Resection and gemcitabine-based chemotherapy	1	
			Resection and etoposide plus cisplatin-based chemotherapy	1	
Pishvaian *et al*[83]	2020	12	Mixed chemotherapy[2]	12	Molecularly guided treatments targeting oncogenic drivers and the DNA damage response and repair pathway require further prospective evaluation, based on these real-world findings
Zong *et al* [84]	2020	11	Resection	4	For pancreatic acinar cell carcinoma, surgery is a potentially curative treatment contributing to long-term survival. It has been confirmed that adjuvant systemic therapy, including chemotherapy and chemoradio-therapy, significantly improves survival compared to surgery alone for resectable ACC. To investigate the role and protocol of perioperative and palliative treatments, additional research with a large sample size is required
			Resection and gemcitabine-based chemotherapy	4	
			Resection and capecitabine	1	
			Resection and mixed chemotherapy[2]	2	
Xu *et al* [85]	2022	22	Resection	6	Although the value of adjuvant chemotherapy remains obscure, fluoropyrimidine-based chemotherapy merits consideration. Fluorouracil-based chemotherapy, such as FOLFIRINOX, may be the preferred treatment for patients with metastasis, but additional research is required due to the small sample size in this study
			Resection and S1-based chemotherapy	3	
			Resection and SOX-based chemotherapy	2	
			Resection and fluoropyrimidine-based chemotherapy	3	
			Resection and AG-based chemotherapy	1	
			Resection and gemcitabine-based chemotherapy	7	
Chen *et al* [86]	2022	26	Resection	11	After radical resection, patients with ACC had a longer overall survival than those with PDAC. ACC is also an aggressive tumor with a similar recurrence-free survival trend to PDAC, necessitating multidisciplinary treatment for resectable ACC disease
			Resection and adjuvant chemotherapy	15	

[1]Not specified in manuscript.

[2]Treatment using more than one anticancer drug including FOLFIRNOX (5-FU + oxaliplatin + leucovorin + irinotecan), CAPOX (oxaliplatin + capecitabine), and S1 (tegafur/gimeracil/potassium).

ACC: Acinar cell carcinoma; PDAC: Pancreatic ductal adenocarcinoma; RT: Radiotherapy; 5-FU: 5-fluorouracil; SOX: S1 + Oxaliplatin; AG: Albumin-bound paclitaxel + gemcitabine; FOLFIRNOX: 5-FU + oxaliplatin + leucovorin + irinotecan.

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ORIGINAL ARTICLE

Basic Study

Expression of the methylcytosine dioxygenase ten-eleven translocation-2 and connexin 43 in inflammatory bowel disease and colorectal cancer

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Abstract

BACKGROUND

Inflammatory bowel disease (IBD) constitutes a substantial risk factor for colorectal cancer. Connexin 43 (Cx43) is a protein that forms gap junction (GJ) complexes involved in intercellular communication, and its expression is altered under pathological conditions, such as IBD and cancer. Recent studies have implicated epigenetic processes modulating DNA methylation in the pathogenesis of diverse inflammatory and malignant diseases. The ten-eleven translocation-2 (TET-2) enzyme catalyzes the demethylation, hence, regulating the activity of various cancer-promoting and tumor-suppressor genes.

AIM
To investigate Cx43 and TET-2 expression levels and presence of 5-hydroxymethylcytosine (5-hmC) marks under inflammatory conditions both *in vitro* and *in vivo*.

METHODS
TET-2 expression was evaluated in parental HT-29 cells and in HT-29 cells expressing low or high levels of Cx43, a putative tumor-suppressor gene whose expression varies in IBD and colorectal cancer, and which has been implicated in the inflammatory process and in tumor onset. The dextran sulfate sodium-induced colitis model was reproduced in BALB/c mice to evaluate the expression of TET-2 and Cx43 under inflammatory conditions *in vivo*. In addition, archived colon tissue sections from normal, IBD (ulcerative colitis), and sporadic colon adenocarcinoma patients were obtained and evaluated for the expression of TET-2 and Cx43. Expression levels were reported at the transcriptional level by quantitative real-time polymerase chain reaction, and at the translational level by Western blotting and immunofluorescence.

RESULTS
Under inflammatory conditions, Cx43 and TET-2 expression levels increased compared to non-inflammatory conditions. TET-2 upregulation was more pronounced in Cx43-deficient cells. Moreover, colon tissue sections from normal, ulcerative colitis, and sporadic colon adenocarcinoma patients corroborated that Cx43 expression increased in IBD and decreased in adenocarcinoma, compared to tissues from non-IBD subjects. However, TET-2 expression and 5-hmC mark levels decreased in samples from patients with ulcerative colitis or cancer. Cx43 and TET-2 expression levels were also investigated in an experimental colitis mouse model. Interestingly, mice exposed to carbenoxolone (CBX), a GJ inhibitor, had upregulated TET-2 levels. Collectively, these results show that TET-2 levels and activity increased under inflammatory conditions, in cells downregulating gap junctional protein Cx43, and in colon tissues from mice exposed to CBX.

CONCLUSION
These results suggest that TET-2 expression levels, as well as Cx43 expression levels, are modulated in models of intestinal inflammation. We hypothesize that TET-2 may demethylate genes involved in inflammation and tumorigenesis, such as Cx43, potentially contributing to intestinal inflammation and associated carcinogenesis.

Key Words: Demethylation; Inflammation-induced carcinogenesis; Ulcerative colitis; Colorectal cancer; Connexins

Core Tip: Chronic inflammation of the colon is a risk factor for colorectal cancer. Intestinal inflammation has been associated with reduced gap junction-mediated intercellular communication and downregulation connexins [mainly connexin 43 (Cx43)]. The involvement of the demethylating enzyme ten-eleven translocation-2 (TET-2) (methylcytosine dioxygenase) in the inflammatory process has motivated the investigation of Cx43 and TET-2 expression in colitis and colorectal carcinoma. *In vitro* and *in vivo* data report on the upregulation of Cx43 in inflammatory states and a downregulation of TET-2. In human samples of colon adenocarcinoma, both TET-2 and Cx43 were downregulated, potentially implicating them in the malignant transformation of inflamed intestinal tissues.

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INTRODUCTION

The gastrointestinal tract is constantly exposed to environmental insults that may potentially lead to pathologies. Inflammatory bowel disease (IBD) is a relapsing and remitting inflammatory disorder affecting distinct parts of the gastrointestinal tract. It comprises two primarily encountered disorders: Crohn's disease and ulcerative colitis, which can be distinguished by their localization in the different

parts of the gut and the endoscopic appearance of the inflamed segment[1-3]. IBD increases the risk of developing colon adenocarcinoma[4], and colitis-associated colon adenocarcinoma remains a fundamental consequence of sustained IBD, possibly because both IBD and colon adenocarcinoma share etiological factors implicated in their development[5].

Gap junctions (GJs), components of junctional complexes, have a potential role in regulating epithelial integrity and function. GJs are formed by the docking of two hemichannels (connexin hexamers) contributed from two adjacent cells, allowing the direct exchange of ions and small signaling molecules (< 2 kDa)[6]. Connexins play a pivotal role in cellular proliferation, differentiation, and function and are recognized as putative tumor-suppressor genes[7-9]. The library of connexin-related diseases has been emerging for many years, and includes various types of syndromes and disorders, such as inflammatory diseases and cancer. Several studies have revealed that the inflammatory response relies in part on connexins and GJ-mediated intercellular communication (GJIC)[10]. More specifically, alteration in the expression of connexin 43 (Cx43) in the gastrointestinal tract is associated with IBD, gastrointestinal infections, and impaired motility[11,12]. We have previously shown an alteration of Cx43 expression and localization, as well as a direct heterocellular communication between intestinal epithelial cells (IECs) and macrophages, which may contribute to IBD pathogenesis[12].

In complex diseases, such as IBD and colon adenocarcinoma, in addition to the heritable component, environmental and epigenetic (DNA methylation and demethylation, histone marks, higher order chromatin structure, *etc.*) factors are likely to influence onset[13]. DNA methylation state is controlled by the interplay between DNA methyltransferases and demethylating enzymes, such as ten-eleven translocation (TET) proteins. Methylation occurs at normally demethylated CpG-rich regions named "CpG islands", of which 70% overlap with human gene promoters, and results in gene silencing[14]. DNA methylation profiles from IBD patients are considerably altered compared to healthy counterparts[15]. Moreover, in IBD-associated colon adenocarcinoma, DNA methylation status was distinct from sporadic colon adenocarcinoma, with different gene expression profiles[16]. DNA methylation is counteracted by demethylation mechanisms, catalyzed by TET enzymes (TET-1, TET-2, TET-3), thus activating gene transcription[17]. Following a series of oxidation reactions by TET enzymes, previously methylated 5-methylcytosine is converted to 5-hydroxymethylcytosine (5-hmC) and further oxidized products that will be recognized and excised by thymine-DNA glycosylase/base enzyme repair pathway to result in an unmethylated cytosine residue[18]. TET genes are mutated in a variety of diseases and cancers. In inflammatory diseases, TET-2 is crucial for the repression of the pro-inflammatory cytokine, interleukin (IL)-6[19], while in several cancers, TET-2 expression is downregulated[20]. In particular, tumors of the digestive tract express different levels of TET enzymes, which are explored as potential factors informing prognosis[21]. Downregulation of TET-1, TET-2, and TET-3 have been reported in gastric cancers with concomitant loss of 5-hmC marks[22] and in colorectal carcinoma[23], with TET-2 transcriptional levels potentially serving as indicators for treatment outcome and disease recurrence [23]. In addition, a recent study reported that the human gene coding for TET-2 contains three promoter elements which are differently regulated in different tissues and developmental stages[24] and may play a role in cancer development.

While many studies have reported the Cx43 promoter to be hypermethylated during the transition from inflammation to cancer[25-27], the expression statuses of Cx43 and TET-2 in IBD and colorectal cancer have not been simultaneously described. This study investigated the effect of inflammation and Cx43 expression levels on the intestinal cell membrane integrity and described TET-2 expression and 5-hmC marks under inflammatory states *in vitro, in vivo*, and in human samples of ulcerative colitis and sporadic colon carcinoma.

MATERIALS AND METHODS

Cell lines and culture conditions

The HT-29 and Caco-2 cell lines derived from colorectal adenocarcinoma tissues were used as models for human IECs. When grown in monolayer, these cells become more differentiated and IEC-like[12,28,29] and are widely used as a model of intestinal transport and pathology, including inflammation. Human embryonic kidney cells (packaging HEK 293T cells) were used for production of viral supernatant for transduction purposes.

In addition, the human monocytic cell line (THP-1) was used as an *in vitro* model for activated macrophages[12,30,31] for the production of conditioned inflammatory medium. After exposure to phorbol myristate acetate (PMA) and lipopolysaccharide (LPS), THP-1 cells become activated; they adhere to the cell culture vessel and show increased transcriptional levels of connexins, Toll-Like Receptor (TLR)-2, TLR-4, NF-κB p65, COX-2, inflammatory cytokines tumor necrosis factor-α (TNF-α), and IL-1β[12]. In this study, suspension THP-1 cells were activated with 50 ng/mL PMA (Sigma-Aldrich, St Louis, MO, United States) for 24 h, followed with 1 μg/mL of LPS (Sigma-Aldrich) for 4 additional hours. When activated THP-1 cells adhered to the cell culture plate, they were washed with PMA- and LPS-free media and left to grow for 72 h. Conditioned media was then collected, filtered, and applied *in vitro* onto colon cell lines to create an inflammatory milieu.

Cells were maintained in complete RPMI-1640 (Sigma-Aldrich) for HT-29 and THP-1 cells or Dulbecco's Modified Eagle's medium (DMEM AQ; Sigma-Aldrich) for HEK 293T cells supplemented with 10% FBS (Sigma-Aldrich), 100 U/mL penicillin G, and 100 µg/mL streptomycin (Sigma-Aldrich). Cells were grown at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

Generation of HT-29 cells upregulating Cx43 gene

The Cx43-pDendra2N construct (Evrogen, Moscow, Russia) was previously cloned into pCSCW lentiviral vectors[12]. The plasmid was then used to transform DH5α competent *Escherichia coli* bacteria, which were then left to proliferate. Plasmid was then isolated and purified using the EndoFree Maxi plasmid purification kit (Qiagen, Hilden, Germany) to be used for transfection along with other plasmids (gag/pol/env) into HEK 293T cells for production of viral supernatant, which carries the Cx43-pDendra2 chimeric proteins. The viral supernatant was used to transduce HT-29 cells. Following the transduction, HT-29 cells were cultured, expanded, and highly positive cells (referred to as HT-29 Cx43D cells thereafter) were sorted using the BD FACSAria™ III sorter (BD Biosciences, Franklin Lakes, NJ, United States). Viral supernatants were obtained from HEK 293T cells and used to transduce HT-29 cells. These cells, named HT-29 Cx43D, were then isolated using a BD Fluorescence-Activated Cell Sorting Aria SORP cell sorter in the single-cell mode. Green fluorescence in HT-29 Cx43D cells attests to the successful upregulation of exogenous Cx43 in these cells.

Generation of HT-29 cells downregulating Cx43 gene using CRISPR/Cas9 system

HT-29 cells knocking down Cx43, referred to as HT-29 Cx43$^-$ cells, were generated using the CRISPR/Cas9 gene-editing system. After cloning the Cx43 target sequence (20 bp) into the guide RNA scaffold of the pX330-CRISPR plasmid, the bacterial transformation was performed as above and 10-15 colonies were picked to check for the correct insertion of the guide RNA by sequencing. Transfection of HT-29 cells with positive clones was performed, and cells were selected with puromycin (1 µg/mL) until isolated colonies were obtained. Several positive clones were validated for Cx43 knockdown at the RNA and protein levels. One specific clone resulted in more than 90% down-regulation of Cx43 mRNA, generating the experimental HT-29 Cx43$^-$ cells. This downregulation was confirmed by western blot and immunofluorescence assays.

Cell growth assay

Parental HT-29, HT-29 Cx43D, and HT-29 Cx43$^-$ cells were seeded in 24-well plates at a density of 25000 cells/cm^2. At 24 h, 48 h, and 72 h, cells were washed with PBS and trypsinized. Viable cells were counted using the trypan blue exclusion dye.

Evaluation of barrier integrity

Trans-epithelial electrical resistance: This method evaluates barrier integrity of epithelial cells grown in monolayer; by describing the impedance of barrier-forming cell cultures. Briefly, electrodes are placed on both sides of the cellular barrier and an electric current is applied. The resulting current established in the circuit is measured and trans-epithelial electrical resistance (TEER) is calculated. The higher the TEER, the better the membrane integrity[32]. In this study, HT-29, HT-29 Cx43D, and HT-29 Cx43$^-$ cells were cultured on Transwell® inserts with 0.4 µm-pore size filters (Corning, Corning, NY, United States). TEER was measured on confluent cells in the presence or absence of 2% dextran sulfate sodium (DSS) using an EVOM voltmeter with an ENDOHM-12 (World Precision Instruments, Sarasota, FL, United States). Electrical resistance was expressed as Ω × cm^2. DSS was applied onto the cells to reproduce *in vitro* the membrane breach it is known to induce *in vivo* and characterize the loss of membrane integrity. TEER was calculated by subtracting the resistance of blank filters from that of filters covered with a monolayer of parental HT-29, HT-29 Cx43D, or HT-29 Cx43$^-$ cells.

Evans blue assay

Epithelial cells form tight junctions that prevent paracellular transit. Evans blue is a dye that strongly binds to serum albumin *in vivo* and *in vitro*, becoming a protein tracer[33]. When Evans Blue is added on the apical aspect of cells in culture, it is retained in this compartment due to the established epithelial barrier. Any perturbation of barrier integrity will result in seeping of Evans Blue to the basolateral aspect of the cells. HT-29, HT-29 Cx43D, and HT-29 Cx43$^-$ cells were grown on transparent PET membrane cell culture inserts with 0.4 µm-pore size (Corning) until confluent (monolayer). Evans Blue solution (Sigma-Aldrich) was prepared in a 1% bovine serum albumin (cell culture grade; GIBCO®, Paisley, Scotland) at a concentration of 170 µg/mL and then filtered through 0.22 µm-filters (Corning®, Wiesbaden, Germany). At confluence, cells were exposed to 2% DSS for 24 h. Cells were then washed twice with PBS and 400 µL of Evans Blue solution were added on top of the cells (seeded in inserts). Plate wells were rinsed and 1 mL of PBS was added into each well. Cells were then incubated at 37 °C. Every 30 min, 200 µL of solution from the well were collected and replaced with fresh 200 µL PBS, for a total of 2 h. The optic density of collected solution was recorded at 630 nm and concentrations of Evans Blue were calculated.

DSS-induced colitis mouse model

This study was approved by the Institutional Animal Care and Utilization Committee of the American University of Beirut (IACUCC# 18-03-476). The acute DSS-induced colitis mouse model was performed as previously reported[34]. The DSS colitis model in mice is a well-established model for IBD. Using this mouse model, studies have shown disruption of the epithelial barrier function with the infiltration of immune cells, as well as an uncommon production of cytokines[35,36]. In addition, DSS has been previously used *in vitro* to induce a cell membrane breach in a monolayer of colon cells, mimicking the intestinal mucosal barrier. DSS exposure resulted in impairment of protein trafficking and alterations in membrane composition in the intestinal Caco-2 cell line[37,38], resembling the intestinal mucosa integrity breach that occurs in IBD.

In this study, carbenoxolone (CBX) was used as a non-specific GJ inhibitor, injected intraperitoneally at the dose of 30 mg/kg every other day. Briefly, adult BALB/c male mice were distributed into the following experimental groups, each comprised of five mice: (1) Control group that received normal drinking water with no CBX injections; (2) CBX group that received normal drinking water with CBX injections starting on day 11; (3) DSS group where mice were exposed to 2.5% DSS in their drinking water for 10 d, followed by normal drinking water as of day 11; and (4) DSS + CBX group that received DSS-containing drinking water for 10 d followed by CBX injections. All four groups were given normal drinking water from day 11 to day 21 (end of experimental duration) and body weights were measured daily. All mice were given standard chow *ad libitum* and were housed in a temperature-controlled environment on a 12-h automated light/dark cycle. On day 21, mice were anesthetized with isoflurane and then euthanized by cervical dislocation. Colon lengths were measured using a ruler and colon tissues were collected for histological and molecular examinations to assess tissue integrity and expression of inflammatory markers, Cx43, and TET-2.

RNA isolation

Cells in culture: Cells were washed with PBS and total RNA was extracted using the RNeasy® Plus mini kit (Qiagen) as *per* manufacturers' instructions.

Cryopreserved tissues: Colon tissues from experimental mice were collected and snap-frozen in liquid nitrogen. RNA was then isolated using TRI reagent (Sigma), as *per* manufacturers' protocol. Since DSS treatment inhibits mRNA amplification from tissues by quantitative polymerase chain reaction (qPCR) by inhibiting the activities of both polymerase and reverse transcriptase, RNA purification for all samples in all conditions was done by the lithium chloride method according to Viennois *et al*[38].

qPCR

One μg of total RNA was reverse-transcribed to cDNA using the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, United States). qPCR was conducted using a homemade SYBR green mix in a CFX96 system (Bio-Rad). Products were amplified using primers that recognize Cx43, TET-2, IL-1β, TNF-α, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1). PCR settings were as follows: A pre-cycle at 95 °C for 5 min followed by 40 cycles consisting of 95 °C for 10 s, 52-62 °C for 30 s, and 72 °C for 30 s with a final extra-elongation at 72 °C for 5 min. The fluorescence threshold cycle value (Ct) was obtained for each gene and normalized to the corresponding GAPDH Ct value. All experiments were carried out in technical duplicates and independently performed at least three times.

Protein extraction and Western blot

Cells in culture: Cells were washed with ice-cold PBS, and scraped on ice in lysis buffer (0.5 M Tris-HCl buffer, pH 6.8; 2% SDS, and 20% glycerol) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland).

Cryopreserved tissues: Snap-frozen mouse colon tissues were homogenized on ice in ice-cold RIPA buffer containing 10% of a 0.5 M Tris-HCl buffer, pH 7.5, 3% of 5 M NaCl, 1% NP-40, 10% sodium deoxycholate, and 1% SDS as well as phosphatase and protease inhibitors.

The lysate was then sonicated. Proteins were quantified, loaded onto 10% SDS-polyacrylamide gels, and subjected to electrophoresis. Migrated proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad) and membranes were blocked with 5% fat-free milk in PBS. Membranes were then incubated with either human or mouse primary antibodies for 3 h at room temperature or overnight at 4 °C. Primary antibodies were hybridized with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit: SC-2357 and anti-mouse SC-2005; Santa Cruz Biotechnology, Dallas, TX, United States) and blots were visualized using an enhanced chemiluminescence detection kit. GAPDH (MAB5476; Abnova, Taipei, Taiwan) and β-actin (A2228; Sigma-Aldrich) were used as loading controls. The quantification of bands was performed using the ImageJ software (United States National Institutes of Health, Bethesda, MD, United States). Immunoreactivity of proteins under study was reported as a ratio of protein-of-interest expression to the housekeeping gene used as loading control.

Table 1 Quantitative polymerase chain reaction human and mouse primer sequences

Gene	Primer sequences, 5' to 3'	Annealing temperature, °C
hCx43	F: CTTCACTACTTTTAAGCAAAAGAG	52
	R: TCCCTCCAGCAGTTGAG	
hTET-1	F: TTCGTCACTGCCAACCTTAG	60
	R: ATGCCTCTTTCACTGGGTG	
hTET-2	F: CACTGCATGTTTGGACTTCTG	60
	R: TGCTCATCCTCAGGTTTTCC	
hTET-3	F: GCCCACAAGGACCAGCATAA	60
	R: CGCAGCGATTGTCTTCCTTG	
hGAPDH	F: TGGTGCTCAGTGTAGCCCAG	52-62
	R: GGACCTGACCTGCCGTCTAG	
mCx43	F: ACAGCGGTTGAGTCAGCTTG	60
	R: GAGAGATGGGGAAGGACTTGT	
mTET-2	F: CAAGGTGTGCTTGGCAATTTT	60
	R: CATCACTGTGCGTCAATCAAGA	
mIl-1β	F: CTCCACCTCAATGGACAGAA	57
	R: GCCGTCTTTCATTACACAGG	
mTNF-α	F: AATGGCCTCCCTCTCATCAGT	57
	R: CCACTTGGTGGTTTGCTACGA	
mGAPDH	F: CATGGCCTTCCGTGTTCCTA	58
	R: CCTGCTTCACCACCTTCTTGAT	

Cx43: Connexin 43; F: Forward; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; h: Human; Il-1β: Interleukin-1β; m: Mouse; R: Reverse; TET: Ten-eleven translocation; TNF-α: Tumor necrosis factor-α.

Specimens from human patients

Archived formaldehyde-fixed paraffin-embedded (FFPE) colon blocks (ulcerative colitis, sporadic colon adenocarcinoma and normal) were obtained from the American University of Beirut Medical Center. All patients' identifiers were kept confidential from the study team.

Histological evaluation

Sections obtained from animals and human samples were stained with hematoxylin and eosin (H&E) for observation under the light microscope (CX41; Olympus, Tokyo, Japan).

Immunofluorescence microscopy

Cells in culture: Cells were grown on coverslips and then fixed either with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 for 20 min for Cx43 probing (SAB4501175; Sigma-Aldrich) or with ice-cold methanol at -20 °C for TET-2 (ab94580; Abcam, Cambridge, United Kingdom) and 5-hmC (ab214728; Abcam) probing before blocking of non-specific binding.

FFPE tissues: Human and murine 5-μm-thick tissue sections were immune-stained using antibodies against Cx43, TET-2, 5-hmC, and CD68 (ab201340, Abcam, United States). Briefly, paraffin blocks were sectioned using a microtome and sections were mounted onto glass slides. Specimens were deparaffinized in xylene and rehydrated using a gradient series of alcohol to water. Antigen retrieval was performed by incubating sections in sodium citrate buffer (pH 6.0) in a steamer for 30 min. Sections were allowed to cool, then washed twice with deionized water before blocking of non-specific binding.

Non-specific binding was blocked with 5% normal goat serum (Chemicon, Burlington, MA, United States) in PBS for 1 h in a humidified chamber and incubated with the primary antibody against Cx43, TET-2, and 5-hmC overnight at 4 °C. Cells were then washed and incubated with IgG-conjugated secondary antibody: Either Texas Red (T862; Life Technologies, Carlsbad, CA, United States) or Alexa Fluor 488 (A11070; Life Technologies) at 1 μg/mL for 1 h at room temperature. Nuclei were counter-stained with 4',6-diamidino-2-phenylindole at 1 μg/mL for 10 min. Coverslips were then mounted onto

the glass slides using Prolong Anti-fade kit (Life Technologies).

Slides were examined under a fluorescence microscope and images were acquired using a 63×/1.46 Oil Plan-Apochromatic objective (on the laser scanning confocal microscope LSM 710, operated by the Zeiss LSM 710 software; Carl Zeiss, Oberkochen, Germany).

Statistical analyses

Numerical values were expressed as mean ± SEM or mean ± SD. The *P* value was determined and considered significant for $P < 0.05$. Differences between experimental groups were assessed using Student *t*-test, one-way analysis of variance (ANOVA) or by two-way ANOVA followed by Tukey's multiple comparisons test.

RESULTS

Characterization of the HT-29 cell model used in this study

In an attempt to investigate TET-2 modulation in the context of different Cx43 expression levels in HT-29 cells, Cx43 was upregulated (HT-29 Cx43D cells) or downregulated (HT-29 Cx43$^-$ cells). Increases and decreases in Cx43 levels were reflected at the translational level: Western blot analysis (Figure 1A) shows upregulation of exogenous Cx43 protein levels in HT-29 Cx43D and loss of endogenous Cx43 protein expression in HT-29 Cx43$^-$ cells compared to parental HT-29 cells ($P < 0.001$). Figure 1B also shows increased expression of Cx43 in HT-29 Cx43D cells and their localization at the cell periphery, where they form GJ plaques, and a decrease in Cx43 levels in HT-29 Cx43$^-$ cells.

Cells manipulated for *Cx43* gene showed differential proliferation rates 48 h and 72 h post-seeding. As depicted in Figure 1C, Cx43 upregulation (HT-29 Cx43D cells) significantly decreased the number of viable cells compared to parental HT-29 cells ($P < 0.05$ at 48 h and $P < 0.001$ at 72 h). However, cells devoid of Cx43 (HT-29 Cx43$^-$ cells) demonstrated a significantly greater proliferation rate compared to parental HT-29 cells at 48 h and 72 h post-seeding ($P < 0.001$). The increased proliferation rate of HT-29 Cx43$^-$ cells was also accompanied by increased migratory potential, as suggested by the wound healing and the invasion assays (Supplementary Figure 1). Compared to parental cells, HT-29 Cx43$^-$ cells seemed more efficient at closing the artificially created gap ($P < 0.05$).

These observations are in accordance with the tumor suppressor role of Cx43, greatly inhibiting the proliferation of HT-29 cells.

Modulation of TET expression in HT-29 cellular subsets

Transcriptional levels of TET-1, TET-2, and TET-3 were evaluated in HT29 cells, manipulated for Cx43 expression. TET-2 specifically demonstrated the highest transcriptional levels between all three TET genes (Figure 1D). TET-2 protein expression was verified by immunofluorescence in parental HT-29 cells (Figure 1D). While TET-1 and TET-3 levels were not significantly different in HT-29 cells expressing lower or higher levels of Cx43, TET-2 levels significantly increased in HT-29 Cx43$^-$ cells compared to parental HT-29 ($P < 0.05$) and to HT-29 Cx43D ($P < 0.001$) cells.

For the remainder of this study, TET-2 expression levels and 5-hmC mark were evaluated given the greater expression of TET-2 in HT-29 cells and the prevalence of loss-of-function mutations of TET-2 in cancer[21,39-41].

Inflammation induces increase in Cx43 and TET-2 expression levels in HT-29 cells

In order to explore a potential pattern of expression for TET-2 and Cx43 in inflammation, parental HT-29, HT-29 Cx43D, and HT-29 Cx43$^-$ cells were screened for TET-2 mRNA and protein expressions, as well as 5-hmC marks by immunofluorescence using an antibody against 5-hmC, a product of the reaction catalyzed by TET-2. In Cx43-expressing cells, the addition of inflammatory media (supernatant from activated THP-1 cells) resulted in upregulation of Cx43 (Figure 2A). Similarly, under inflammatory conditions, all three cellular subsets upregulated their TET-2 expression ($P < 0.05$) at the transcriptional and translational levels (Figure 2B). Interestingly, HT-29 cells down-regulating Cx43 showed higher expression of TET-2 compared to parental cells or cells overexpressing Cx43 (Figure 2B). The 5-hmC marks also increased with increased TET-2 levels in HT-29 Cx43$^-$ cells. Levels of TET-2 and 5-hmC both increased in cells exposed to inflammation, as shown by quantified immunofluorescence micrographs (Figure 2C). Changes in TET-2 expression were less pronounced in HT-29 Cx43D cells than in HT-29 Cx43$^-$ cells. This trend was also evident with the 5-hmC marks (Figure 2C) where increased expression of TET-2 correlated with increased amounts of 5-hmC.

In order to strengthen these findings, experiments were performed on Caco-2 cells. Levels of Cx43 and TET-2 were also upregulated in Caco-2 cells exposed to inflammatory medium (Figure 2D), with a five-fold increase in Cx43 levels ($P < 0.001$) and two-fold increase in TET-2 levels ($P < 0.01$).

Downregulating Cx43 contributes to the disruption of epithelial membrane integrity

Parental HT-29, HT-29 Cx43D, and HT-29 Cx43$^-$ cells were grown as monolayers and exposed to 2% DSS

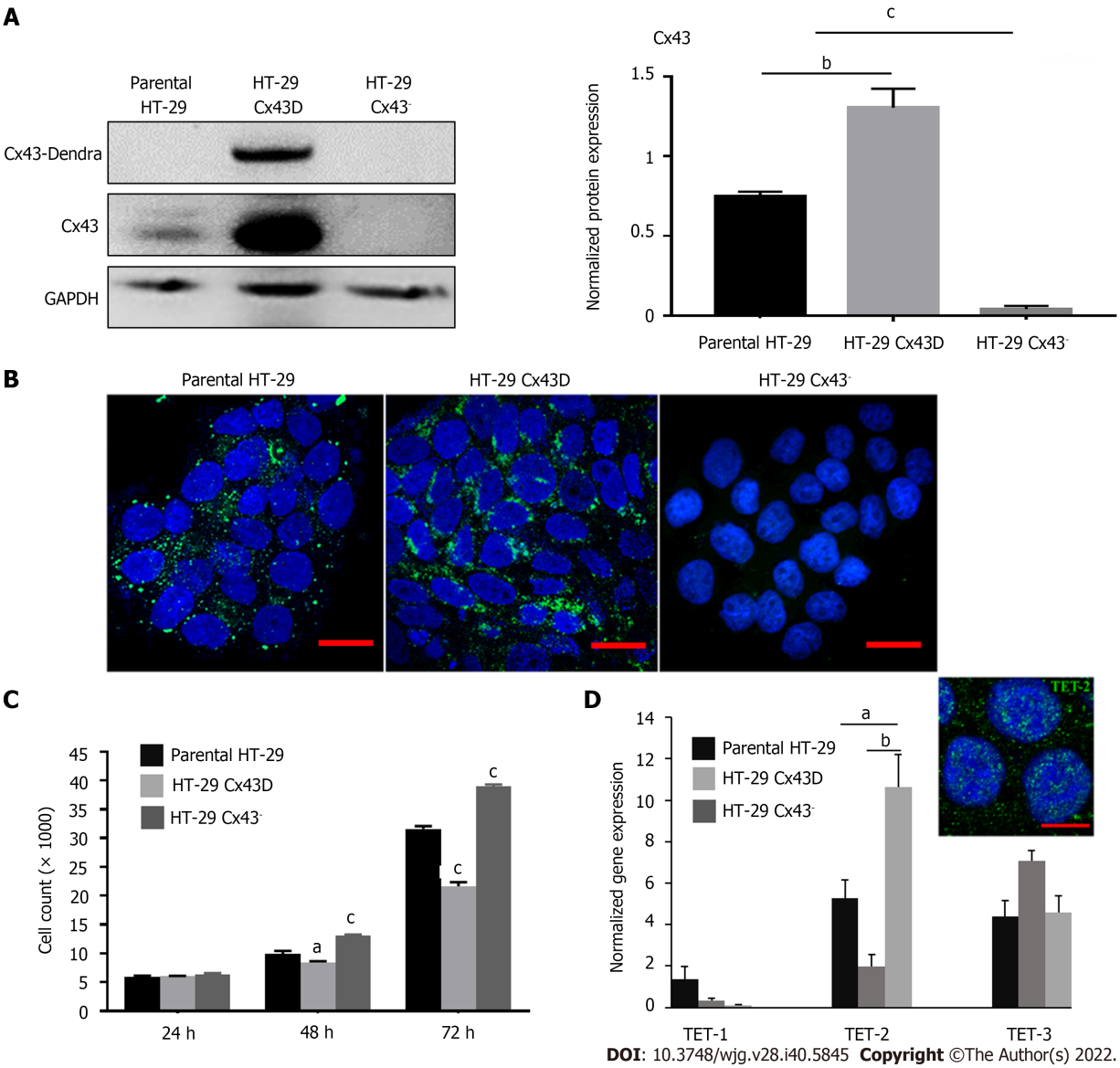

Figure 1 Characterization of the *in vitro* HT-29 cell model. A: Western blot of connexin 43 (Cx43) protein expression in parental HT-29, HT-29 Cx43-Dendra (Cx43D), and in HT-29 Cx43- cells. Bar graphs display mean densitometric analysis (of three independent experiments) after normalizing protein expression to glyceraldehyde 3-phosphate dehydrogenase (GAPDH); B: Immunofluorescence micrographs of parental HT-29 and HT-29 Cx43- cells, as well as a fluorescent micrograph of HT-29 cells transduced with the GFP-Cx43 construct. Highly GFP positive cells were sorted using BD-Fluorescence-Activated Cell Sorting cell sorter, generating the HT-29 Cx43D. Scale bar 10 μm; C: Cells were counted at three different time points (24, 48 and 72 h), using the trypan blue dye exclusion assay. Compared to parental HT-29 cells, HT-29 Cx43D cells had a slower proliferation rate and HT-29 Cx43- cells a faster proliferation rate at 48 and 72 h; D: Histograms show normalized gene expression of ten-eleven translocation (TETs) in parental HT-29, HT-29 Cx43D and HT-29 Cx43- cells, as detected by quantitative polymerase chain reaction (qPCR). TET-2 was the highest expressed TET in HT-29 cells, and cells with differential Cx43 expression also had significantly different TET-2 expression levels. The inset is an immunomicrograph of TET-2 in HT-29 C43D cells. Scale bar 5 μm. Results are presented as means ± SEM of three independent qPCR runs in duplicates. One-way ANOVA, [a]$P < 0.05$; [b]$P < 0.001$; [c]$P < 0.0005$. Cx43: Connexin 43; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

for 24 h to evaluate if differential Cx43 levels would have any bearing on DSS-induced membrane integrity breach. DSS was previously shown to impair IEC membrane integrity *in vitro*[42] and it was used in our study to induce membrane injury similar to that observed upon chronic inflammation. Figure 3A shows that DSS resulted in decreased cell contacts and increased spaces between cells (red arrows), indicating loss of membrane integrity. The TEER assay was performed to assess how Cx43 modulation affects membrane integrity in the presence of DSS. Figure 3B shows that, in the absence of DSS, differentially expressed Cx43 had no major impact on epithelial barrier integrity, with an average TEER of 20 Ω × cm^2 in parental and in HT-29 Cx43- cells and 23 Ω × cm^2 in HT-29 Cx43D cells. However, the addition of DSS resulted in a significant decrease in TEER values by approximately 37% in parental cells and 50% in HT-29 Cx43- cells ($P < 0001$). Therefore, overexpression of Cx43 in HT-29 Cx43D cells seemed to be protective against DSS-induced loss of membrane integrity (Figure 3B).

The Evans Blue permeability assay was then performed to evaluate the monolayer integrity under DSS exposure and in cells with different Cx43 expression status. The level of cell permeability or "leakage" was correlated to the concentration of Evans Blue measured at the bottom of the well. As

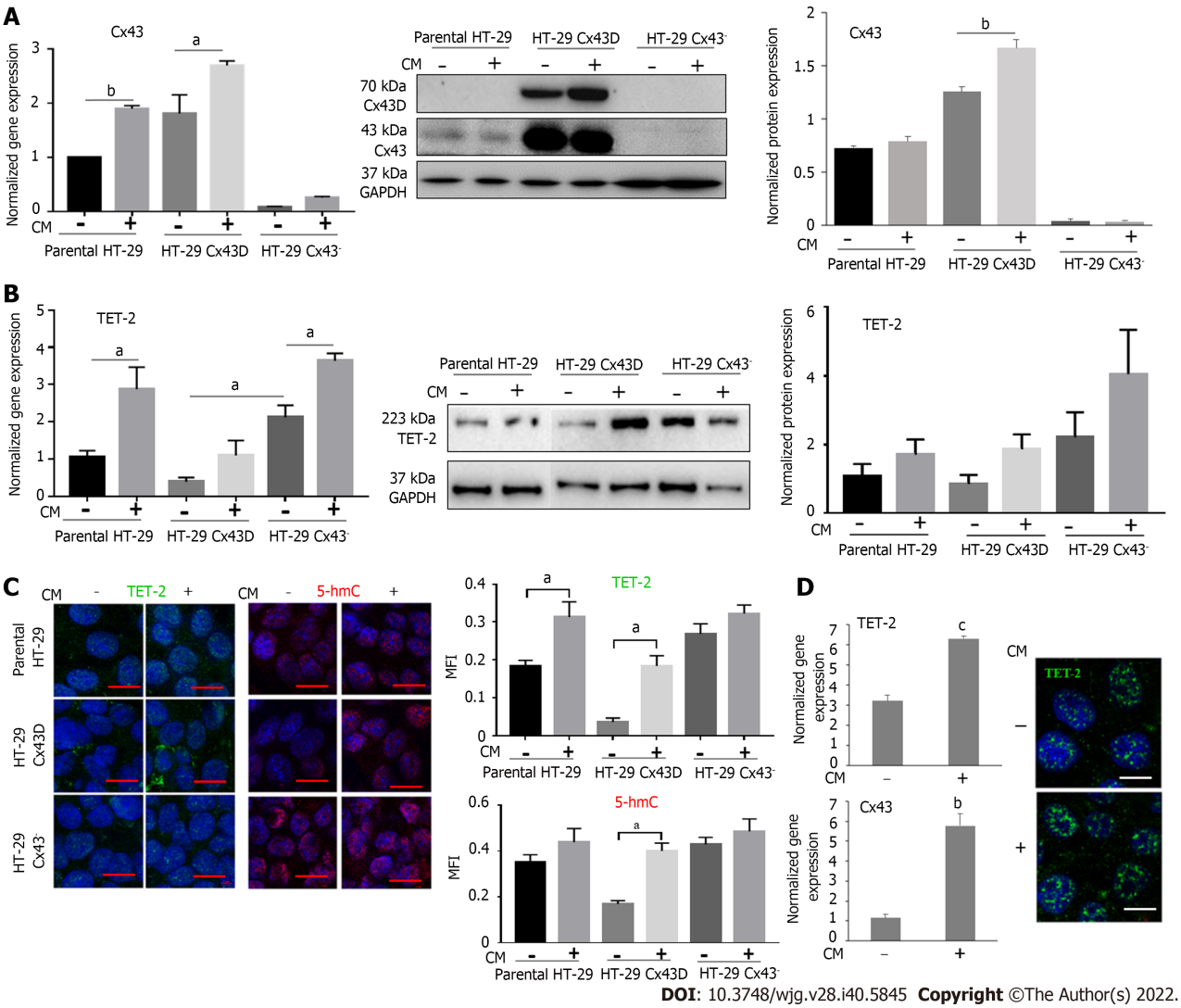

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Figure 2 Connexin 43 and ten-eleven translocation-2 expression increases in cells exposed to inflammation. Parental HT-29, HT-29 connexin 43-Dendra (Cx43D), and HT-29 Cx43$^-$ cells were exposed to inflammatory conditioned media (CM) obtained from activated THP-1 cells for 24 h. A: Histograms show the normalized gene expression of Cx43, as detected by quantitative polymerase chain reaction. Cx43 mRNA levels increase in parental HT-29 and HT-29 Cx43D, in the presence of inflammatory media. Western blot of endogenous Cx43 and exogenous Cx43D protein expression in parental HT-29, HT-29 Cx43D, and in HT-29 Cx43$^-$ cells. Densitometric analysis (ratio of protein-of-interest to loading control band intensity) shows a slight increase in Cx43 levels upon exposure to inflammation; B: Bar graphs show ten-eleven translocation-2 (TET-2) transcriptional levels increase in all three HT-29 cellular subsets when exposed to CM. Western blots of TET-2 and densitometric analysis shows increased protein levels of TET-2 in CM-treated cells; C: Immunofluorescence images showing TET-2 expression and 5-hmC marks in parental HT-29, HT-29 Cx43D, and HT-29 Cx43$^-$ cells. Bar graphs in the right panel reflect mean fluorescence intensity of at least five different fields acquired from three different experiments. Levels and activity of TET-2 increase in all CM-exposed cells. Scale bar 5 μm; D: Bar graphs display levels of Cx43 and TET-2 in Caco-2 cells exposed to CM. Fluorescent micrographs show increased levels of TET-2 in the nucleus upon exposure to CM. Scale bar 5 μm. Experiments were repeated at least three different times. One-way ANOVA, $^aP < 0.05$; $^bP < 0.001$; $^cP < 0.0005$. TET-2: Translocation-2; Cx43: Connexin 43; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; MFI: Mean fluorescence intensity.

shown in Figure 3C, after 60 min of incubation with Evans Blue, the concentration of the dye significantly and rapidly increased in DSS-treated parental HT-29 ($P < 0.05$) and HT-29 Cx43$^-$ cells ($P < 0.0001$). However, within the two-hour timeframe, cells overexpressing Cx43 (HT-29 Cx43D cells) had not leaked substantial levels of Evans Blue dye.

DSS was subsequently used to reproduce the murine colitis model to evaluate modulation of Cx43 and TET-2 levels in the inflamed colons of mice. Figure 3D shows that upon exposure of HT-29 cells to DSS *in vitro*, levels of Cx43 and TET-2 vary. Specifically, in Cx43-expressing cells, DSS exposure results in upregulation of Cx43 ($P < 0.001$). Levels of TET-2 also significantly increase in all HT-29 cellular subsets ($P < 0.01$).

In summary, *in vitro* results suggest that inflammation leads to upregulation of Cx43 and of TET-2 in HT-29 cells. TET-2 upregulation was more pronounced in HT-29 cells devoid of Cx43. Moreover, Cx43 knockdown rendered HT-29 cells more sensitive to DSS-induced membrane integrity breach, in favor of a role of Cx43 protein in the maintenance of epithelial barrier integrity. Modulation of Cx43 and TET-2 *in vitro* displayed similar trends whether cells were exposed to conditioned inflammatory media

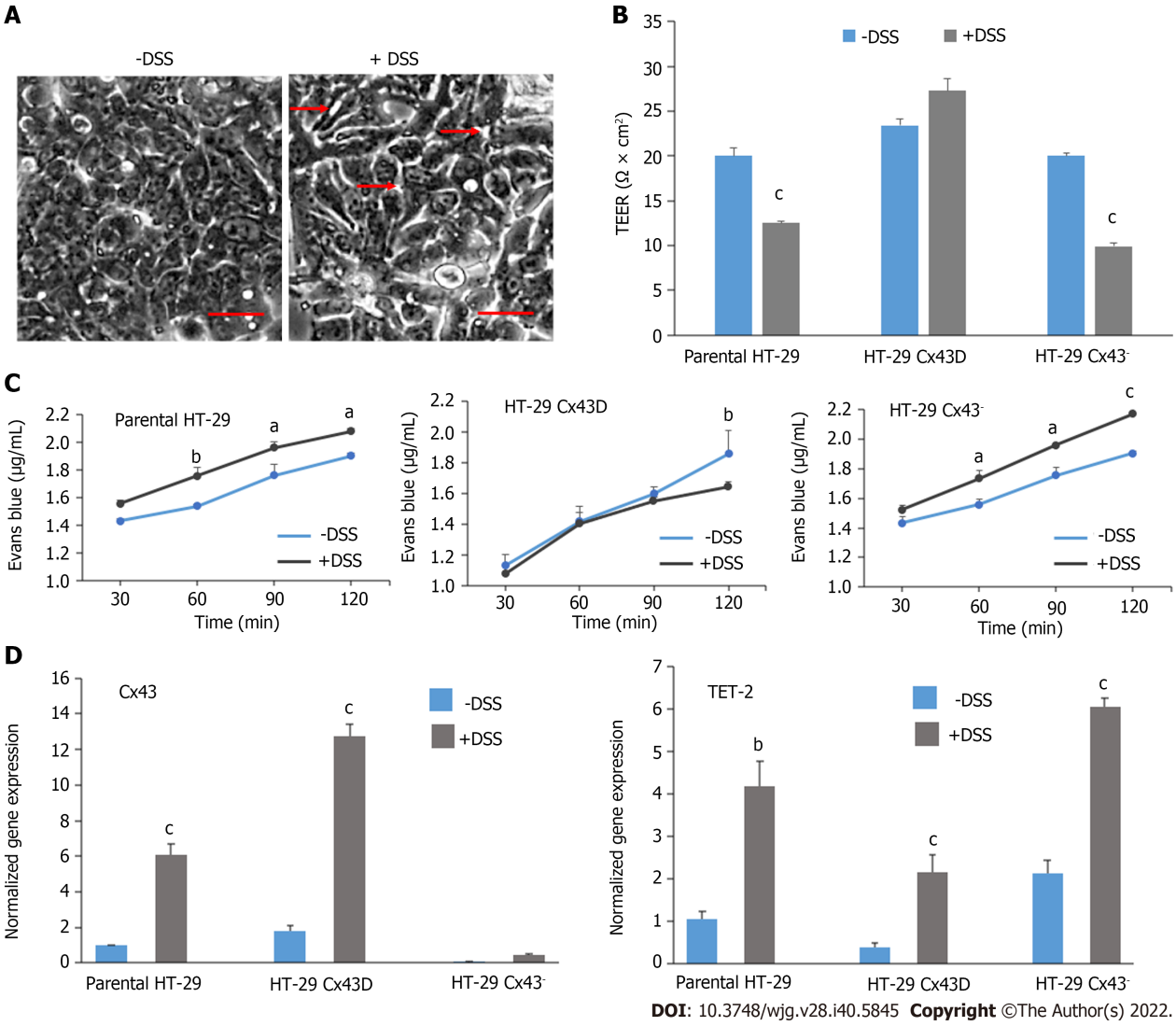

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Figure 3 Dextran sulfate sodium treatment disrupts the integrity and permeability of intestinal epithelial barrier, notably in HT-29 connexin 43- cells. A: Light microscopy images of HT-29 cells in the presence or absence of 2% dextran sulfate sodium (DSS). Scale bar 50 μm; B: The membrane integrity of parental HT-29, HT-29 connexin 43-Dendra (Cx43D), and HT-29 Cx43- cells in the presence or absence of 2% DSS was measured by trans-epithelial electrical resistance (TEER). Bar graphs indicate average TEER measurements; C: Membrane permeability was evaluated by Evans Blue permeability assay in all three cell subsets. Levels of Evans Blue that crossed are measured by spectrophotometry and are plotted as means over time; D: Quantitative polymerase chain reaction data show that transcriptional levels of Cx43 and ten-eleven translocation-2 (TET-2) significantly increase in DSS-treated cells. All experiments have been repeated at least three times. Two-way ANOVA, $^{a}P < 0.05$; $^{b}P < 0.001$; $^{c}P < 0.0005$. Cx43: Connexin 43; DSS: Dextran sulfate sodium; TEER: Trans-epithelial electrical resistance.

(obtained from activated THP-1 cells) or to DSS (subsequently used in the colitis mouse model).

An established and widely used DSS-induced colitis mouse model was reproduced[34] to evaluate Cx43 and TET-2 expression. A subset of mice was also administered CBX, a GJ inhibitor, to examine TET-2 expression modulation in varying functional states of GJs.

DSS-induced colitis in mice is attenuated by CBX-induced GJ blockade

The experimental design was as described in Figure 4A, where each group comprised five mice. Body weight was recorded daily, throughout the 21-d experimental duration. Figure 4B reflects weight changes; while mice in the control group (Group 1) consistently gained weight over the 21-d experiment, mice exposed to CBX alone (Group 2) maintained their baseline weight throughout the experiment. On the other hand, DSS-exposed mice lost a moderate amount of weight until day 10, after which they were switched to normal drinking water (Group 3) and their average weight picked up to almost control group levels. Mice in the DSS group who were injected with CBX starting day 11 recovered some of the weight lost until day 10, but did not reach the normal weight recorded in the control group.

At the end of the experimental duration, mice were euthanized and their colons examined and collected for measurement purposes and a biopsy was used for histological and molecular analyses.

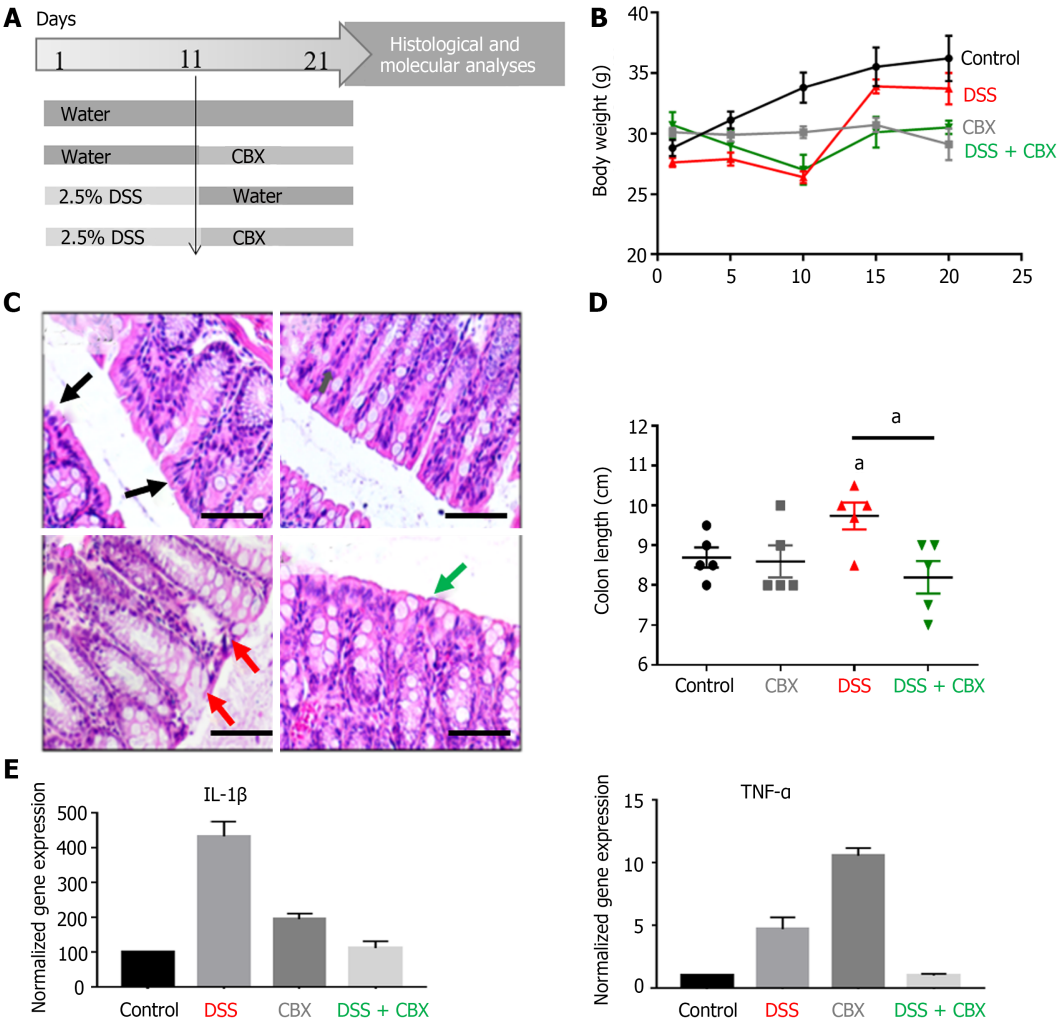

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Figure 4 Dextran sulfate sodium induces inflammation in mice and carbenoxolone restores the normal phenotype. A: A flowchart schematizing the *in vivo* experimental design. BALB/c male mice were distributed into four experimental groups, each comprised of five mice: (1) Control group that received normal drinking water with no carbenoxolone (CBX) injections; (2) CBX group that received normal drinking water with CBX injections starting day 11; (3) Dextran sulfate sodium (DSS) group where mice were administered with 2.5% DSS in their drinking water for 10 d but not subjected to any CBX injections; and (4) DSS + CBX group that received DSS-containing drinking water as well as CBX injections. Mice were given 30 mg/kg CBX *via* intraperitoneal injections from day 11 every other day to the end of the experiment; B: Variation of animal body weight over 21 d. DSS-exposed mice lost weight. Significance in body weight change was detected between days 4 and 12 in DSS-treated mice, and days 11 and 21 in CBX-injected mice only; C: Hematoxylin and eosin images showing the architecture of mice colon tissues from all experimental groups. Arrows indicate representative sites in each experimental condition. Scale bar 50 μm; D: Colon length was measured at the end of the experiment. DSS-treated mice showed significantly longer colons compared to the control group; E: Bar graphs showing the normalized gene expression of interleukin-1β and tumor necrosis factor-α in mouse colon tissues, as detected by quantitative polymerase chain reaction. Experiments were repeated five times and results are displayed as means ± SEM. One-way ANOVA, $^{a}P < 0.05$. IL: Interleukin; CBX: Carbenoxolone; DSS: Dextran sulfate sodium; TNF-α: Tumor necrosis factor-α.

Histological examination of H&E stained sections of colons showed a disruption of epithelial barrier as well as an increase in infiltrating cells in crypts of colons from DSS-treated mice compared to control. CBX-treated animals seem to have retained (albeit not fully) some normalcy (Figure 4C). DSS-treated mice showed significantly longer colons compared to the control group ($P < 0.05$). CBX significantly reduced the length of DSS-treated colons to approximately normal levels ($P < 0.05$) (Figure 4D).

Levels of inflammatory cytokines, IL-1β and TNF-α, previously shown to be modulated in IBD models[12,38,43], were evaluated by qPCR. In accordance with weight changes and colon length indicative of intestinal damage, DSS-treated mice of Group 3 had increased expression of both IL-1β and TNF-α, compared to control Group 1 ($P < 0.05$) (Figure 4E).

These data propose that CBX alleviates DSS-induced inflammation in mice, as demonstrated by normalized colon length and histology, as well as decreased levels of inflammatory mediator transcripts in colon tissues.

DSS-induced inflammation increases Cx43 and TET-2 expression in vivo

Colon tissues obtained from mice on day 21 (end of experimental duration) were processed for total

RNA and protein extraction. Cx43 expression was significantly higher in tissues from mice exposed to DSS both at the transcriptional (Figure 5A, $P < 0.0001$) and translational levels (Figure 5A, middle and right panels). However, CBX injections restored Cx43 mRNA and protein levels back to control levels. Not only did GJ inhibition attenuate the inflammatory state in the colon, it also led to downregulated Cx43 expression.

Levels of TET-2 were also evaluated in the colon tissues obtained from mice in all four experimental groups. Gene expression analysis pointed to an increase of TET-2 mRNA levels in the CBX-treated group (Group 2, Figure 5B, $P < 0.001$). In the tissues of mice from Group 4 (DSS + CBX), TET-2 levels were similar to those in the DSS group (slightly, but not significantly, higher than control levels). The same pattern was observed at the protein level (Figure 5B, middle and right panels). Changes in TET-2 levels were accompanied by changes in Cx43 levels, which increased in DSS-treated (inflamed) colons and decreased upon addition of CBX (Figure 5C). Inflammation was further underscored by immunostaining using CD68 antibody. As shown in Figure 5D, levels of infiltrated $CD68^+$ cells (macrophages) increased significantly by six-fold in colons of DSS-treated mice as opposed to those of control or CBX-treated mice alone ($P < 0.0001$). In accordance with CBX effects on inflammatory mediators and histological aspects, CBX injections following DSS exposure resulted in a decreased amount of macrophages. Sections from colons of mice in the DSS group also showed considerable levels of TET-2 protein in epithelial cells with neighboring macrophages, both indicative of an active inflammatory process. TET-2 levels increased further in colons of CBX-treated mice. In addition, and consistent with transcriptional and translational data, CBX-treated mice had upregulated TET-2 protein levels in their colons (Figure 5D). Immunofluorescent micrographs reflect TET-2 level increase in mucosal cells, though not to the same extent as qPCR data; due to the contribution of TET-2 mRNA from $CD68^+$ macrophages in the vicinity of IECs.

These data indicate that inhibiting GJs in inflamed tissues mitigates DSS-induced inflammation. In addition, the increase in Cx43 expression upon exposure to DSS is accompanied by an increase in TET-2 protein expression levels, and disabling GJs by CBX is paralleled by a significant increase in TET-2 levels. Data therefore suggest that, under inflammatory conditions (DSS), GJ inhibition by CBX restores Cx43 levels down to control. In accordance with *in vitro* data where HT-29 $Cx43^-$ cells had upregulated TET-2 levels, chemical inhibition of Cx43 *in vivo* also was accompanied by increased TET-2 levels.

Collectively, *in vitro* and *in vivo* data indicate that under inflammatory conditions, levels of both TET-2 and Cx43 increase.

Cx43 levels increase and TET-2 levels decrease in colons of patients with ulcerative colitis

In an attempt to correlate *in vitro* and *in vivo* findings with clinical data, FFPE colon biopsies from normal non-dysplastic non-inflamed colons ($n = 7$), ulcerative colitis ($n = 5$), and colon adenocarcinoma ($n = 7$) patients were obtained anonymously. Sections were stained with H&E for light microscopy, as well as with antibodies against CD68 (macrophage marker), Cx43, and TET-2 for immunofluorescence analyses.

Histological staining exposed a clearly disorganized intestinal mucosal layer in ulcerative colitis and sporadic colon adenocarcinoma tissues as compared to normal tissues (Figure 6A). The disrupted architecture was due to extensive immune activity in the tissues underlying the mucosa. Figure 6B shows $CD68^+$ macrophages infiltrating tissues from ulcerative colitis patients ($P < 0.0001$), underscoring the inflammatory profile. Tissues obtained from patients with sporadic adenocarcinoma of the colon also displayed macrophage foci, but to a much lesser extent than ulcerative colitis tissues ($P < 0.001$). Although comparable to control in terms of fluorescence intensity, $CD68^+$ cells in the adenocarcinoma specimen were not uniformly distributed in the tissue.

Moreover, Cx43 and TET-2 expression and localization were evaluated by immunofluorescence (Figure 6C and D). Consistent with data obtained *in vitro* (Figures 2 and 3) and *in vivo* (Figure 5), Cx43 protein levels were significantly increased in ulcerative colitis (*i.e.* inflamed) tissues ($P < 0.005$) and sharply decreased in adenocarcinoma. Unlike *in vitro* and *in vivo* data that reflect parallel patterns of Cx43 and TET-2 expression in inflammatory conditions, Cx43 level increase in ulcerative colitis was accompanied by a decrease in TET-2 expression ($P < 0.001$). This observation, however, resembles the *in vitro* scenario illustrated in Figure 2. In fact, while TET-2 expression had increased upon exposure of cells to an inflammatory milieu, Cx43 upregulation in HT-29 Cx43D cells was associated with lower overall TET-2 expression compared to parental cells, and Cx43 knockdown resulted in elevated TET-2 levels.

DISCUSSION

In various pathologies, including inflammation and cancer, the alteration of the expression, regulation, and association of junctional complexes with other proteins have been reported. Epigenetic methylation and demethylation processes are also major players in a cell's malignant transformation. Specifically, GJ-forming Cx43 and demethylating enzyme TET-2 have both been documented to be impaired in inflammation and cancer[10-12,19,20].

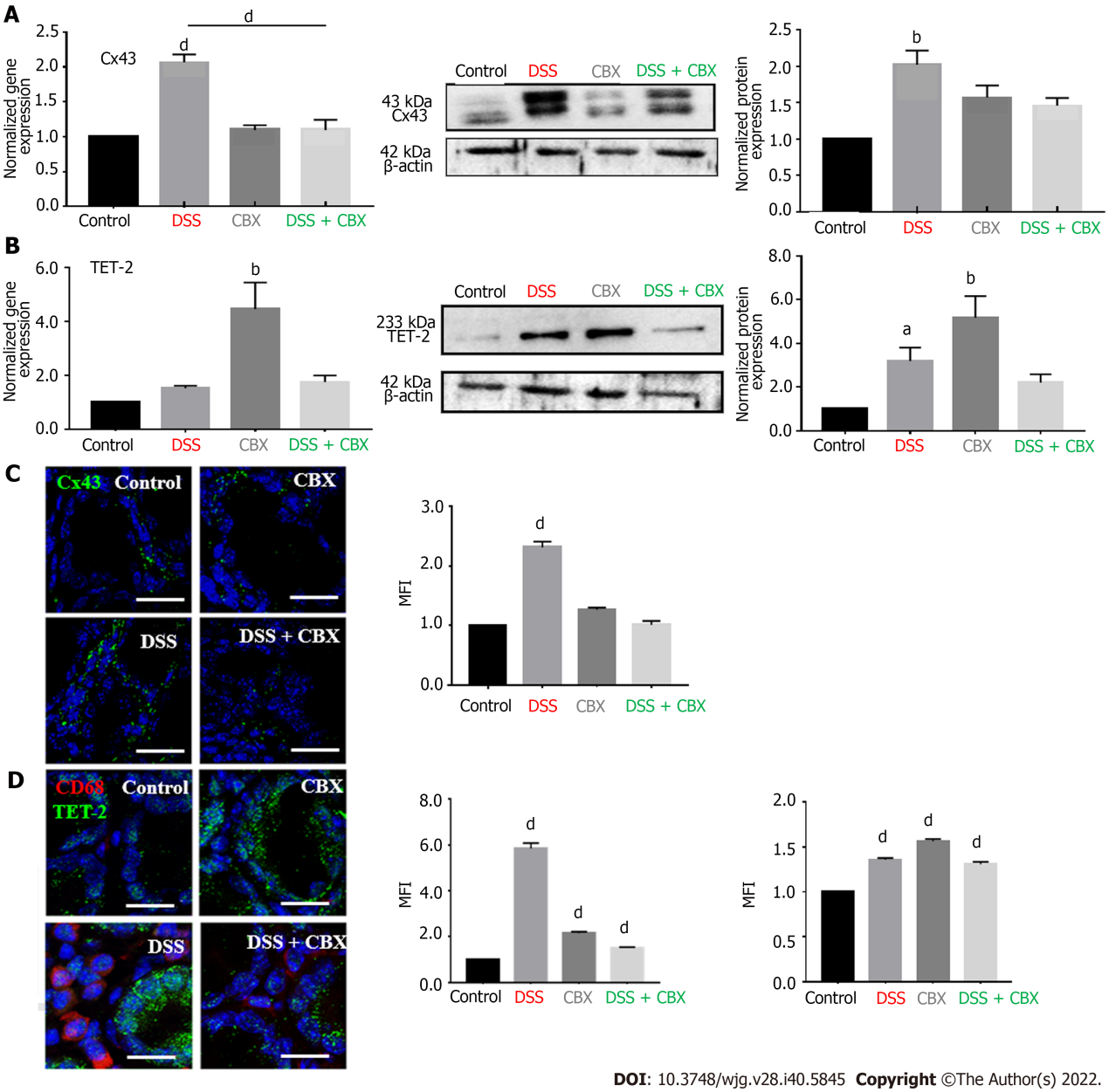

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Figure 5 Dextran sulfate sodium increases connexin 43 and ten-eleven translocation-2 expressions *in vivo*. A: Left panel: Histograms show the normalized gene expression of connexin 43 (Cx43) in mice colon tissues, as detected by quantitative polymerase chain reaction (qPCR). Middle panel: Western blot of Cx43 protein in mouse colon tissues. Right panel: Densitometric analysis of protein expression after normalization to β-actin. Dextran sulfate sodium (DSS)-treated mice had the highest expression of Cx43 in their colons; B: Left panel: Histograms display the normalized gene expression of ten-eleven translocation-2 (TET-2) in mouse colon tissues, as detected by qPCR. Middle panel: Western blot of TET-2 protein expression in mouse colon tissues. Right panel: Densitometric analysis of protein expression after normalization to β-actin. DSS exposure resulted in enhanced TET-2 transcription and carbenoxolone inhibition of gap junctions significantly increased TET-2 expression; C: Immunofluorescence images of colon tissues stained for Cx43 with the mean fluorescence intensity (MFI) analysis. Scale bar 20 μm; D: Immunofluorescence images of mouse colon tissues stained for CD68 and TET-2. Histograms reflect the MFI analysis. High levels of CD68$^+$ cells correlated with greater expression of Cx43 and TET-2 in DSS-exposed mice. Scale bar 10 μm. Experiments were repeated five times. One-way ANOVA, $^aP < 0.05$; $^bP < 0.005$; $^dP < 0.0001$. MFI: Mean fluorescence intensity; CBX: Carbenoxolone; DSS: Dextran sulfate sodium.

Studies have shown that the inflammatory response relies in part on intercellular communication mediated by channel-forming connexins. The role of GJ-mediated signaling in the initiation of inflammation is mainly through the transfer of ATP between neighboring cells. ATP molecules are then released in the extracellular environment where they act as signaling molecules that activate purinergic receptors, amplifying the inflammatory response[10]. Wong *et al*[11] partly elucidated the role of Cx43 in intestinal epithelial barrier injury. TLR activation in the disrupted intestinal epithelium translates intracellularly into Cx43 transcription, translation, post-translational modification, and assembly into connexons. GJIC regulates intestinal epithelial function during both acute and chronic inflammation [11]. Furthermore, Al-Ghadban *et al*[12] demonstrated a role of GJs in the pathogenesis of IBD by a direct communication between IECs and macrophages enhanced by basement membrane degradation [12]. Long-term IBD associates with amplified risk of colon carcinogenesis, and the deregulation of

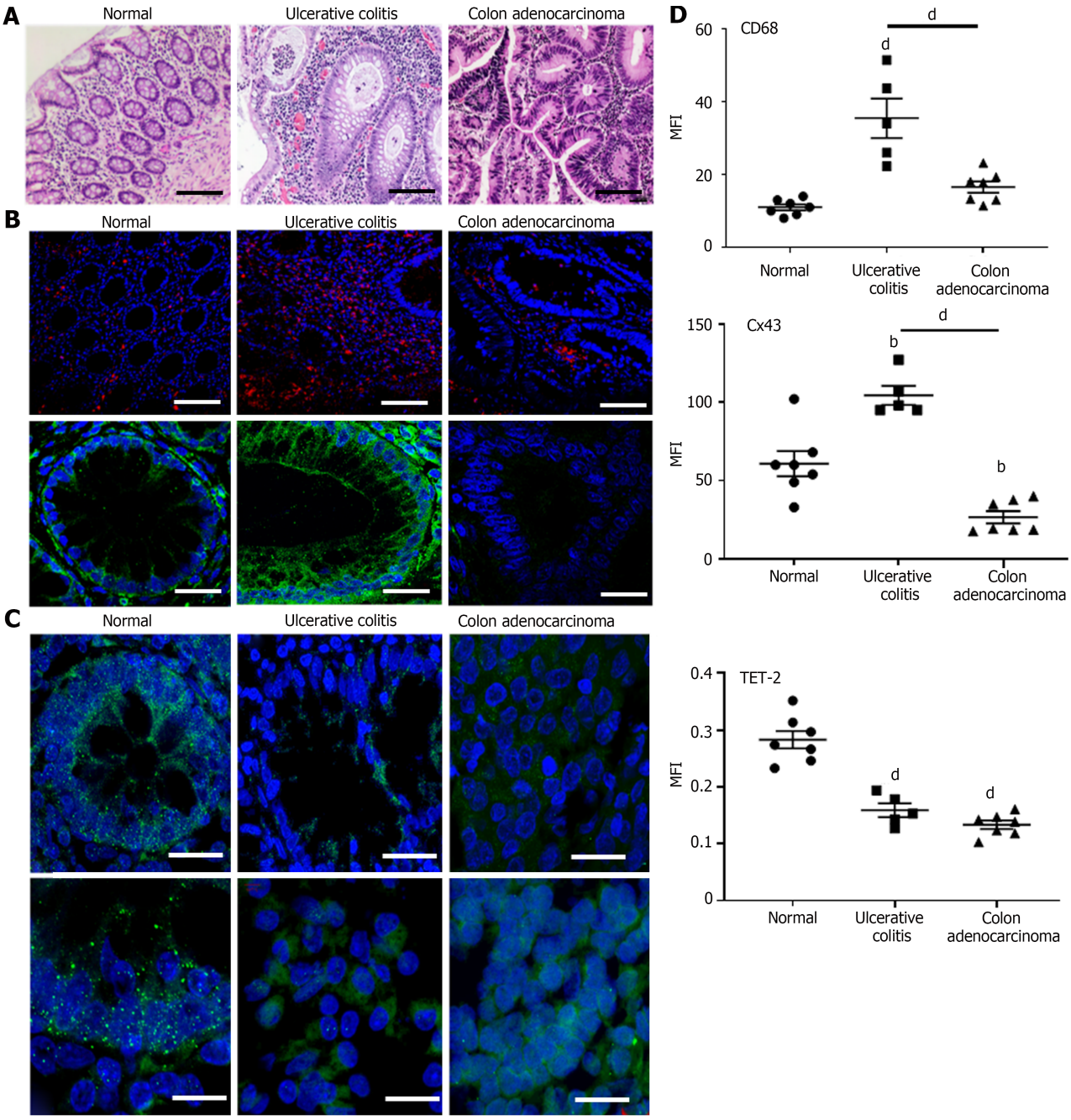

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Figure 6 Modulation of connexin 43 and ten-eleven translocation-2 expression in human colon tissues under pathological conditions. A: Hematoxylin and eosin images of human colon tissues. Compared to normal (histopathologically unchanged) samples, tissues from inflamed (ulcerative colitis) and sporadic malignant colons show disorganization of colon crypts and infiltration of immune cells within the lamina propria. Scale bar 50 μm; B: Immunofluorescence images of $CD68^+$ cells (macrophages) and connexin 43 (Cx43) show that Cx43 expression increases in inflamed tissues. Scale bar 10 μm; C: Immunofluorescence images of ten-eleven translocation-2 (TET-2) in human colon tissues (upper panel: Low magnification; lower panel: Higher magnification). TET-2 protein was minimally detected in ulcerative colitis and colon adenocarcinoma tissues. Scale bar 10 μm; D: Mean fluorescence intensity (MFI) analyses reflecting the expression of CD68, Cx43, and TET-2. One-way ANOVA, $^bP < 0.005$; $^dP < 0.0001$. MFI: Mean fluorescence intensity; TET: Ten-eleven translocation.

connexin expression is regarded as one of the hallmarks of different cancers. Shilova *et al*[44] reviewed *in vitro* and *in vivo* studies that point at a correlation between the loss of connexin expression and cancer onset. In fact, downregulation of Cx26, Cx32, and Cx43 accompanies the development of human bladder cancer, hepatocarcinoma, and breast cancer, respectively[44]. A recent study on breast cancer proposed that Cx43 upregulation in a triple negative breast cancer cell line led to a reversal of its mesenchymal phenotype *in vitro* and to a decreased metastatic potential *in vivo*[7]. While connexin downregulation is observed in many cancers, elevated Cx43 levels have also been associated with increased malignancy. In a hepatocellular carcinoma cell line, Cx43 was shown to enhance malignancy by inhibiting Cx32-mediated GJIC[45], and, in prostate cancer, Cx43 promoted invasion and metastasis[46]. Variability of data on connexin involvement in cancer onset and progression warrants more research to identify the

underlying factors that dictate a tumor-suppressing or tumor-enhancing activity of connexins. Epigenetic regulation of various genes could explain the differential expression of tumor-suppressor and tumor-enhancer genes in inflammation and cancer onset and progression.

The role of demethylating enzyme TET-2 in IBD and colorectal adenocarcinoma has not been fully described. In fact, TET proteins have mostly been studied in hematological malignancies. TET-1 was characterized as a partner gene to the *Mixed-Lineage Leukemia* gene in acute myeloid leukemia (AML), and the *TET-2* gene was described in a study on the myelodysplastic syndrome[47]. Recent investigations report that frequent point mutations in the *TET-2* gene can lead to the truncation of the resulting protein and loss of enzymatic activity, reflected by reduced global levels of the 5-hmC marks in AML patients[41,48]. However, although mutations in the *TET-1* and *TET-3* genes are rare in hematological malignancies compared to *TET-2*, both still exhibit a tumor-suppressor role: TET-1 in B-cell lymphomas [41] and TET-3, together with TET-2, in murine aggressive myeloid cancer[49]. Mutations and reduced expression of TET proteins were also observed in solid tumors, and decreased translational levels of TETs seem like an important hallmark of different cancers, including cancers of the digestive system[50-52].

In addition to carcinogenesis, a study by Zhang *et al*[19] shows an anti-inflammatory role for TET-2, which, under inflammatory conditions, repressed IL-6 transcription in dendritic cells and macrophages [19]. Moreover, TET-2-deficient bone marrow-derived dendritic cells and macrophages had high transcriptional levels of IL-6 upon LPS challenge. Another study also reported an increase of TET-2 transcriptional levels in colon tissues from patients with IBD[53].The present study examined the expression of Cx43 and TET-2 in an *in vitro* model of colon epithelium, where HT-29 cells were used in their parental state or with upregulated or knocked-out Cx43 in the presence or absence of inflammation (induced by DSS or by the addition of an inflammatory medium). A DSS-induced colitis murine model was used to describe how inflammation modulates the expression of Cx43 and TET-2. Data were then compared to Cx43 and TET-2 expression levels in tissues obtained from patients with ulcerative colitis or sporadic colorectal cancer.

Results from this study indicate that knockout of Cx43 in the HT-29 intestinal cell line leads to disruption of membrane integrity exacerbated by the DSS chemical insult. In Cx43-deficient cells and in mice that were subjected to chemical inhibition of GJs by CBX, loss of GJs was paralleled with increased TET-2 expression, further intensified in the presence of inflammation. In fact, inflammation triggered an upregulation of Cx43 and TET-2 expression in all cell subsets, which is in accordance with the literature [19,47]. An exploratory analysis of human colon samples obtained from ulcerative colitis patients revealed an increase in Cx43 and a decrease in TET-2 protein levels accompanied by the disruption of colon architecture. Though these findings are not aligned with the overall trend implicating TET-2 in intestinal inflammatory conditions, little is known about characteristics of patients whose colon biopsies were examined, including potential pharmacological agents that could lead to TET-2 degradation *via* one of the four pathways reviewed by Cong *et al*[54] in October 2020: A caspase-dependent pathway, calpain1-promoted degradation of TET-2, proteasome-dependent degradation, and p53-facilitated autophagy of TET-2 proteins[54]. Moreover, TET-2 seems to be implicated in both initiation of the inflammatory process by activating innate pro-inflammatory signaling pathways and its resolution by inducing the repression of pro-inflammatory mediators[55]. Therefore, the exact status of TET-2 in the colon of IBD patients remains to be elucidated, in light of multiple factors governing post-transcriptional and post-translational TET-2 regulation. In specimens from sporadic colon adenocarcinoma, both TET-2 and Cx43 proteins were considerably downregulated, underscoring the tumor-suppressor role of Cx43.

In the context of IBD (specifically ulcerative colitis) and colon cancer, we propose that Cx43 upregulation in inflamed colons could attenuate or slow down the onset of malignancy. Therefore, loss of Cx43 in tissues from colon cancer could be attributed to a malignant switch, turning off tumor-suppressor genes and activating tumor-promoting genes. The presence of $CD68^+$ cells (macrophages) was concomitant with loss of TET-2 expression in the colon adenocarcinoma samples. This could be explained by an immune-active phenotype in tumors, with tumor-associated macrophages having little to no TET-2 expression[55]. A 2018 study reported increased levels of TET-2 in T lymphocytes from colorectal tumor tissues, leading to demethylation and activation of FOXP3 and regulation of regulatory T cell (Treg) function[56], further implicating TET-2 in immune-related processes. In particular, TET-2 has been reported to regulate the innate immune response[57,58]. While it serves to activate the inflammatory response, TET-2 has also been implicated in inflammation resolution, where in response to IL-1/RMyD88 signaling, TET-2 downregulates the expression of inflammatory cytokines, such as IL-6 in innate myeloid cells and IL-1β in macrophages by recruiting HDACs for histone deacetylation[19,57]. This property might explain low levels of TET-2 in human ulcerative colitis samples where unresolved inflammation can be attributed to persistent inflammatory cytokines.

TET-2 status and role in digestive cancers remain unclear and variable, depending on the cancer site and grade, but also on possible mutations that could lead to cancer progression and resistance to chemotherapy, as recently reviewed[21].

CONCLUSION

Data presented in this manuscript show that exposure of intestinal cells to inflammation is associated with Cx43 and TET-2 upregulation *in vitro* and *in vivo*. We propose that, as part of its potential anti-inflammatory role and through its demethylating activity, TET-2 might be responsible for promoting the expression of anti-inflammatory genes and for indirectly repressing the expression of pro-inflammatory genes under inflammatory conditions. Similarly, we hypothesize that the demethylating TET-2 enzyme might promote the expression of tumor-suppressor genes (among others, Cx43) in the inflamed colon. Extrapolating from observations made on human colon samples, a malignant switch could happen in chronically inflamed colons. This malignant switch could be associated with downregulation of TET-2 (and potentially other TET enzymes[20,23]) in ulcerative colitis, possibly resulting in the hypermethylation of genes that are relevant in the context of carcinogenesis, such as Cx43. Although we present evidence on the modulation of TET-2 expression and we reiterate the role of Cx43 in intestinal inflammation, further investigation is under way to more solidly explore the mechanism of action behind a potential interplay between Cx43 and TET-2 that might, at least partly and indirectly, bridge chronic inflammation (such as in IBD) and IBD-induced carcinogenesis in the colon.

ARTICLE HIGHLIGHTS

Research background

Patients with inflammatory bowel disease (IBD) have a higher propensity to acquire colorectal cancer. The link between inflammation and cancer has long been established; however, the molecular players in the switch to cancer are still poorly defined.

Research motivation

In previous work, we have demonstrated a potential role for connexin 43 (Cx43) in inflamed intestinal cells. Recognizing the tumor-suppressor role of Cx43 in several cancers, we set out to explore whether the loss of Cx43 is associated with the switch to carcinogenesis. One potential mechanism for downregulating Cx43 expression is through methylation. We hypothesized that ten-eleven translocation-2 (TET-2), a demethylating enzyme, previously described to have a role in inflammation, may be involved in this process.

Research objectives

Using cell culture, a colitis animal model, and archived human tissues, we assessed the expression of both Cx43 and TET-2 in intestinal inflammation. Specific objectives include: (1) Assessment of the expression levels of Cx43 and TET-2 under inflammatory conditions; (2) Assessment of the expression and activity of TET-2 in HT-29 cell lines with up- or down-regulated for Cx43 expression; (3) Reproduction of a colitis mouse model and assessment of Cx43 and TET-2 levels in colons of mice; and (4) Explore Cx43 and TET-2 expression levels in archived biopsies obtained from patients with ulcerative colitis and colon adenocarcinoma.

Research methods

This study employed several modalities to verify the hypothesis, which include intestinal epithelial cell (IEC) line modified for Cx43 expression grown under inflammatory conditions. A dextran sulfate sodium-induced colitis mouse model was reproduced and tissues from different experimental conditions were analyzed. Gene expression profile, protein expression levels, morphology and cellular localization were described. In addition, archived formalin-fixed paraffin-embedded tissues were sectioned and evaluated for Cx43 and TET-2 expression.

Research results

In vitro, TET-2 expression was elevated under inflammatory conditions and even more so in HT-29 cells devoid of Cx43. The barrier function of IECs was breached when Cx43 levels were down-regulated. These results were corroborated in the murine colitis model. In archived biopsies from ulcerative colitis patients, Cx43 expression was upregulated compared to non-inflamed counterparts. In sporadic colon adenocarcinoma sections, both TET-2 and Cx43 expression levels were decreased.

Research conclusions

Under inflammatory conditions, levels of Cx43 and of the demethylating enzyme TET-2 are upregulated. Through demethylation, TET-2 would turn on the expression of several factors involved in inflammation (presumably Cx43 included). When TET-2 levels were diminished in sporadic colon adenocarcinoma, we also observed that Cx43 was downregulated, which may indicate a role of TET-2 in shutting down Cx43 and its tumor-suppressing potential.

Research perspectives

In vitro, manipulation of TET-2 levels in intestinal cells may yield further insight into the mechanism of action. Methylation studies will also be undertaken. The animal model will be expanded to allow for the development of colon carcinoma, and timed evaluation of molecular players will be performed. More stringent criteria will be implemented for prospective tissue collection from non-inflamed subjects, patients with IBD, and with IBD-associated colorectal cancer.

FOOTNOTES

Author contributions: El-Harakeh M, Saliba J, Haidar M, and Sharaf Aldeen K equally contributed to the study; El-Sabban M conceived and designed the study; Hashash JG, Awad MK, Shirinian M, and El-Sabban M provided the material and resources for the experiments; El-Harakeh M, Haidar M, Sharaf Aldeen K, and El Hajjar L performed the experiments; Saliba J and El-Harakeh M analyzed the data and wrote the manuscript; Shirinian M and Hashash JG critically reviewed the manuscript; Saliba J and El-Sabban M revised the manuscript; All authors read and approved the final version of the manuscript.

Institutional review board statement: Archived formaldehyde-fixed paraffin-embedded (FFPE) colon blocks (ulcerative colitis, sporadic colon adenocarcinoma and normal) were obtained from the American University of Beirut Medical Center (AUBMC). All patients' identifiers were kept confidential and all samples were anonymous, hence exempted from ethics committee approval.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the American University of Beirut, No. 18-03-476.

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ORIGINAL ARTICLE

Basic Study

Curcumin alleviates experimental colitis *via* a potential mechanism involving memory B cells and Bcl-6-Syk-BLNK signaling

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Abstract

BACKGROUND

Immune dysfunction is the crucial cause in the pathogenesis of inflammatory bowel disease (IBD), which is mainly related to lymphocytes (T or B cells, including memory B cells), mast cells, activated neutrophils, and macrophages. As the precursor of B cells, the activation of memory B cells can trigger and differentiate B cells to produce a giant variety of inducible B cells and tolerant B cells, whose dysfunction can easily lead to autoimmune diseases, including IBD.

AIM

To investigate whether or not curcumin (Cur) can alleviate experimental colitis by regulating memory B cells and Bcl-6-Syk-BLNK signaling.

METHODS

Colitis was induced in mice with a dextran sulphate sodium (DSS) solution in drinking water. Colitis mice were given Cur (100 mg/kg/d) orally for 14 consecutive days. The colonic weight, colonic length, intestinal weight index, occult blood scores, and histological scores of mice were examined to evaluate the curative effect. The levels of memory B cells in peripheral blood of mice were measured by flow cytometry, and IL-1β, IL-6, IL-10, IL-7A, and TNF-α expression

in colonic tissue homogenates were analyzed by enzyme-linked immunosorbent assay. Western blot was used to measure the expression of Bcl-6, BLNK, Syk, and other signaling pathway related proteins.

RESULTS

After Cur treatment for 14 d, the body weight, colonic weight, colonic length, colonic weight index, and colonic pathological injury of mice with colitis were ameliorated. The secretion of IL-1β, IL-6, TNF-α, and IL-7A was statistically decreased, while the IL-35 and IL-10 levels were considerably increased. Activation of memory B cell subsets in colitis mice was confirmed by a remarkable reduction in the expression of IgM, IgG, IgA, FCRL5, CD103, FasL, PD-1, CD38, and CXCR3 on the surface of $CD19^+CD27^+$ B cells, while the number of $CD19^+CD27^+$ IL-10^+ and $CD19^+CD27^+$ Tim-3^+ B cells increased significantly. In addition, Cur significantly inhibited the protein levels of Syk, p-Syk, Bcl-6, and CIN85, and increased BLNK and p-BLNK expression in colitis mice.

CONCLUSION

Cur could effectively alleviate DSS-induced colitis in mice by regulating memory B cells and the Bcl-6-Syk-BLNK signaling pathway.

Key Words: Curcumin; Experimental colitis; Memory B cell; Bcl-6; BLNK

Core Tip: As the precursor of B cells, the activation of memory B cells can trigger the activation of B cells, thus producing numerous inducible B cells and tolerant B cells, whose dysfunction can easily result in inflammatory bowel disease (IBD). Also, the disorder of humoral immune function mediated by memory B cells has a critical position in the pathogenesis of IBD. As an immune suppressant, curcumin (Cur) has a therapeutic role in treating many immune diseases, such as IBD and rheumatoid arthritis. Meanwhile, it has proven efficacy in experimental colitis mice and patients with ulcerative colitis (UC), while the disturbances in memory B cells have been observed in IBD. However, few studies have explored whether Cur treatment of colitis is closely related to memory B cells. In the present research, our results indicated that Cur effectively alleviated DSS-induced UC in mice *via* a potential mechanism involving memory B cells and the Bcl-6-Syk-BLNK signaling pathway.

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INTRODUCTION

Curcuma longa L, also named turmeric, is a perennial herb pertaining to the genus Curcuma in the *Zingiberaceae* of the *Scitamineae,* native to China, Indonesia, and India. According to the existing literature, *Curcuma longa L* can promote Qi, break blood stasis, facilitate menstruation, and relieve pain [1]. The bioactive natural polyphenols extracted from the rhizome of *Zingiberaceae* are called curcumin (Cur) [1,7-bis(4-hydroxy-3-methoxyphenyl)- 1,6-heptadiene-3,5-dione]. They are also known as diferuloylmethane, and their molecular formula $C_{21}H_{20}O_6$ accounts for 75% to 80% of curcumin compounds[2] (a structure is shown in Figure 1A)[3]. According to recent carefully designed clinical studies, with an excellent safety profile, Cur has the potential effect of prevention and/or management of various diseases including inflammatory bowel disease, dysentery, chronic enteritis, gastrointestinal syndrome, and so on, due to its anti-oxidant, anti-apoptotic, and anti-inflammatory properties[4]. In addition, many preclinical studies have indicated that Cur has perfect anti-cancer properties to inhibit carcinogenesis and the proliferation of various cancer cells[5], including prostate, oral epithelial leukemia, hepatic, breast, and colon cancers in human and animals, and the probable mechanisms are closely related to modulating the activation of a variety of cellular signals as apoptosis and angiogenesis [6].

As a chronic nonspecific inflammatory disorder, inflammatory bowel disease (IBD), classified into ulcerative colitis (UC) and Crohn's disease (CD), is characterized by recurrent abdominal pain, diarrhea, anemia, bleeding, and weight loss. However, the pathogenesis of IBD is still unclear, and is closely

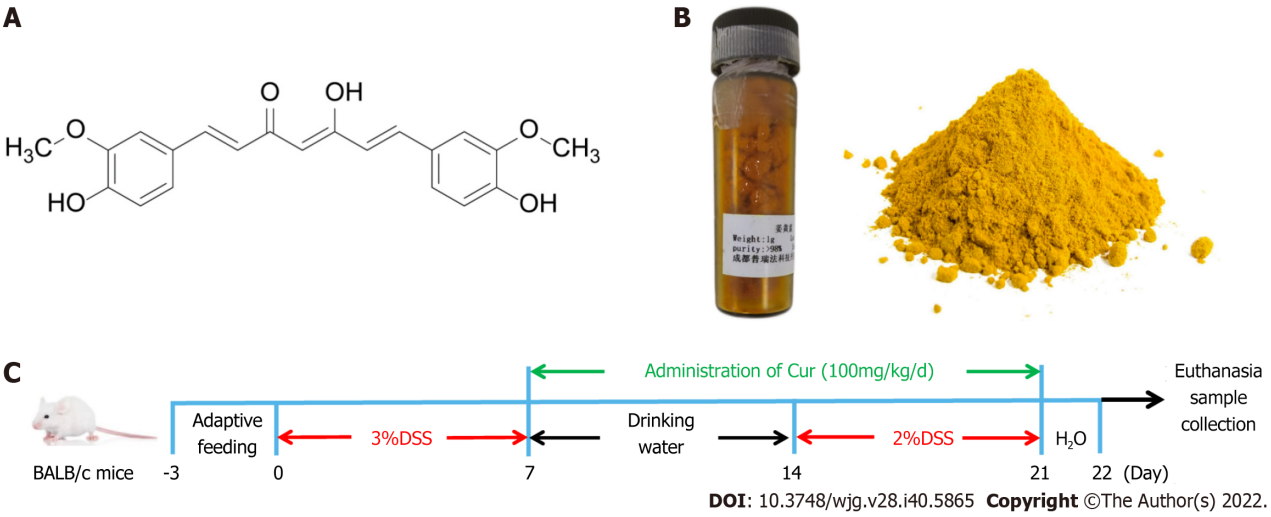

Figure 1 Drugs and protocols used in the study. A: Molecular structural formula of curcumin (Cur); B: Cur used in the experiment; C: Colitis induction and Cur administration. The experiment lasted for 22 d, including 3 d of adaptive feeding, 0-7 d of treatment with 3% DSS treatment with drinking water, and 7 d of free drinking water. Animals were divided into four groups: Control (n = 10), DSS (n = 10), DSS + Cur (100 mg/kg/d, n = 10), and Ctrl + Cur (100 mg/kg/d, n = 10) groups.

associated with heredity, environment, infection, immunity, and so on[7]. Among these, immune system dysfunction has been regard as the main cause of IBD, which is mainly involving abnormal lymphocytes (including T and B cells, including memory B cells), macrophages, mast cells, and activated neutrophils[8]. As a precursor to B cells, the activation of memory B cells can trigger the activation of B cells, thus producing numerous inducible B cells and tolerant B cells, whose dysfunction predisposes to autoimmune diseases, including IBD[9]. Pararasa *et al*[10] reported a decrease in $CD27^+IgD^-$ memory B cells in the blood of patients with IBD, but a concomitant increase in $CD27^-IgD^-$ memory B cells in gut-associated lymphoid tissue, which suggests that the disorder of humoral immune function mediated by memory B cells has a very important role in the pathogenesis of IBD.

Many previous studies have demonstrated that Cur can be used to effectively treat UC patients and animals. An endoscopic study revealed that NCB-02 enema (containing 140 mg Cur) used once per day for 8 wk could ameliorate the disease symptoms in patients with UC[11]. In an animal study, Cur administration effectively alleviated the colonic mucosal inflammation, restored colonic length, and reduced colonic weight and colonic damage[10]. Potential mechanisms of Cur for treating UC are diverse, including regulating immune function (such as regulating the function and level of T cells, regulatory T cells, memory T cells, macrophages, and dendritic cells), inhibiting the secretion of IL-2, IL-6, IL-12, IL-17, and TNF-α[12], downregulating the expression of costimulatory molecules[13], inhibiting the chemotaxis of chemokines and neutrophils, and inducing antioxidant effect[14]. Cur may induce apoptosis in colorectal cancer as it induces reactive oxygen species production, suppressing NF-κB signaling activation and cyclooxygenase-2 expression, activating caspase 3 and c-Jun N-terminal kinases (JNK), and inducing the release of cytochrome C. It is known that Cur can promote the activation of caspase 3, caspase 8, caspase 9, Bax, and poly (ADP-ribose) polymerase (PARP) to induce apoptosis in HCT-116 colon cancer cells[15]. Furthermore, it also exerts its effect on other autoimmune diseases by effectively regulating memory B cells. Elham and colleagues[16] found that Cur achieved effective results for autoimmune diseases such as RA and systemic lupus erythematosus (SLE) by targeting B lymphocyte stimulating factor (BLYS), which is an important cytokine for memory B cell proliferation. However, it is unclear whether Cur exerts a therapeutic effect on IBD by regulating the subsets or function of memory B cells. Therefore, in this study, DSS-induced colitis model, a classic animal model replicating human UC, was used to evaluate and explore the mechanism and therapeutic effect of Cur on IBD. Flow cytometry and other experiments were performed to explore the possible mechanisms and the changes in memory B cell subsets after Cur treatment.

MATERIALS AND METHODS

Mice

BALB/c male mice, aged 8-9 wk and weighing 20-22 g, were purchased from the Hunan Silaike Jingda Experimental Animal Co. Ltd. (Changsha, China; Animal Certificate No. SCXK 2016-0002). Mice were provided with standard food and water *ad libitum*, and lived under specific pathogen-free (SPF) conditions at the temperature of 23 °C ± 2 °C and relative humidity of 55% ± 10% with alternating light/darkness for 12 h. They were acclimatized for 3 d prior to the start of the experiment. All animal

experiments were conducted and performed in line with the regulations and guidelines of the Jiangxi University of Chinese Medicine Animal Care and Use Committee and the AAALAC and the IACUC (Permit No. JZLLSC2021-196).

All experimental mice were randomly divided into the following four groups: Control group, in which mice were fed normal food without DSS or drug treatment; Control + Cur group, in which normal mice were given Cur treatment; DSS group, in which mice with DSS-induced colitis were administrated with Cur; DSS + Cur group, in which mice with DSS-induced colitis were treated with Cur.

DSS-induced experimental colitis

As previously described, mice with colitis were freely given a 3% (w/v) solution of DSS (Batch No. 160110) in drinking water for 7 d (Figure 1C), then with sterile drinking water for 7 d, and finally with 2% (w/v) DSS for 7 d. Meanwhile, mice in the control group were given tap water. Cur was provided by Chengdu Purifa Technology Development Co., LTD (Chengdu, China; Batch No. PRF10052344, purity > 98%), as shown in Figure 1B. In order to better dissolve the drug, Cur was dissolved in 1.5% sodium carboxymethylcellulose. According to our previous study[17], mice in the DSS + Cur group and Control + Cur group were administrated with Cur (100 mg/kg/d) by gavage for 14 consecutive days, while mice in the Control and DSS groups were administrated with an equal volume of saline starting from day 8. The mice were weighed daily to evaluate body weight changes, and their fecal character, stool bleeding, and live state were monitored[18].

Histological evaluation

After the last administration, the mice were fasted for 12 h. All mice were weighed before anesthesia with sodium pentobarbital (20 mg/kg *i.p.*). After euthanasia, the whole colons were rapidly separated on the ice box, and their length and weight were measured to calculate the colon weight index (CWI) as [colon weight/body weight] × 100%, and the colon length index (CLI) as (colon weight/colon length) × 100%. The distal colon tissue was fixed in 4% paraformaldehyde for 72 h. After embedding in paraffin to cut 4 μm thick sections, the sections were dehydrated in an ethanol gradient series and stained with hematoxylin-eosin for histopathological analysis. Finally, the slices were sealed with neutral gum. And then the colonic pathological changes were observed and double-blindly scored by two pathologists under a microscope (Leica, Wetzlar, Germany, Product model: DM2500). According to the previous standards, a composite score was computed and totalized with inflammatory cell infiltration (scores 0-3), mucosal damage (scores 0-3), crypt damage (scores 0-4), and regeneration (scores 0-4)[19].

Flow cytometry

In order to isolate peripheral lymphocytes, 500 μL of peripheral blood from each mouse was collected and treated with 1 mL of lysing buffer (BD Biosciences, Franklin Lakes, NJ, United States) and incubated for 15 min in the darkness to remove red blood cells. The cell suspension was combined with an Fcγ receptor-blocking mAb (CD16/32; BioLegend, San Diego, CA, United States) for 15 min at 4 °C. Later on, the cells were detected for surface antigens by labelling with BV510 rat anti-mouse CD19 (Lot No. BD562956), AF488 rat anti-mouse CD27 (Lot No. BD124222), PE-A rat anti-mouse IgM (Lot No. BD553409), BV421 rat anti-mouse IgG (Lot No. BD742475), APC rat anti-mouse IL-10 (Lot No. BD505010), BV421 rat anti-mouse IgA (Lot No. BD743293), APC rat anti-mouse FCRL5 (Lot No. BD340305), PE-A rat anti-mouse CD103 (Lot No. BD562772), BV421 rat anti-mouse FasL (Lot No. BD740054), APC rat anti-mouse PD-1 (Lot No. BD562671), BV421 rat anti-mouse CXCR3 (Lot No. BD126529), and PE-A rat anti-mouse Tim-3 (Lot No. BD119704), which were purchased from BD Bioscience (San Jose, CA, United States). Finally, the single-cell suspensions were analyzed with a FACS Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, United States). Gates were set for the quadrant markers based on negative populations and isotype controls. FlowJo VX software (TreeStar, San Carlos, CA, United States) was used to analyze all data to differentiate the memory B cell subgroups.

Enzyme-linked immunosorbent assay (ELISA)

One hundred micrograms of mouse colon tissue was homogenized by adding 1000 μL of RIPA lysis buffer (Cell Signaling Technology, Danvers, MA, United States). The samples were incubated at 4 °C for 1 h, ultrasonically homogenized, and centrifuged at 13000 rpm for 10 min, and the supernatant was taken to obtain colonic tissue homogenate. The total protein in colonic tissue homogenate was quantified with a total protein detection kit (Aidlab Biotechnologies Co., Ltd., Beijing, China; Lot No. PP0102). According to the manufacturer's instructions, commercial ELISA kits (Thermo Fisher Scientific, Waltham, MA, United States) were used to detect the secretion of IL-1β (Lot No. BMS6002TEN), IL-6 (Lot No. BMS603-2), IL-10 (Lot No. 88-7105-88), IL-35 (Lot No. BMS616), IL-17A (Lot No. BMS6001), and TNF-α (Lot No. BMS607-3).

Western blot analysis

Colon tissue homogenate was prepared and protein concentration determination was performed

according to the above methods. First, equal amounts of colonic mucosa protein were resolved by polyacrylamid gel electrophoresis and transferred onto PVDF membranes, which were then blocked with 3% bovine serum albumin (BSA) solution for 1 h at room temperature and then incubated with the following primary antibodies overnight at 4 °C: BLNK (Abcam, ab32418, 1:500), p-BLNK (Abcam, ab174837, 1:1000), Syk (Abcam, ab40781, 1:1000), p-Syk (Abcam, ab300410, 1:1000), CIN85 (1:1000), Bcl-6 (Abcam, ab19011, 1:1000), and anti-GAPDH (Abcam, ab181602, 1:1000) antibodies. Next, the corresponding HRP-coupled secondary antibody (Abcam, ab205718, 1:5000) was added and incubated for 2 h at room temperature, and the ECL hypersensitive solution (Thermo, Rockford, IL, United States) was used to visualize the protein blotting. Photographs were taken by using the Highly Sensitive Chemiluminescence Imaging system (UVP ChemStudio 515; Analytik Jena, Jena, Germany). Image-Pro Plus 6.0 software (Media Cybernetic, Bethesda, MD, United States) was used to quantify the images, and the gray value of the band and the ratio to that of the internal reference GAPDH was used for statistical analysis.

Statistical analysis

GraphPad Prism 7.0 software (San Diego, CA, United States) was used to analyze the difference between groups by performing independent samples *t*-test, one-way analysis of variance, least-significant difference test, and Turkey test for multiple comparisons. All data are presented as the mean ± SE. $P < 0.05$ was considered statistically significant.

RESULTS

Cur alleviates DSS-induced colitis

Many studies have shown that mice with DSS-induced colitis may present diarrhea, abdominal pain, bloody stool, weight loss, shortened colon length, increased colon weight, and other symptoms[20]. Under the microscope, altered colonic gland structure, a decrease in the number of goblet cells, massive infiltration of inflammatory cells in the mucosa and submucosa, and obvious erosive ulcer in DSS-induced colitis can be observed[21]. In our pilot study, DSS-induced colitis mice exhibited significant weight loss from day 4 to day 21 of the experiment (Figure 2A), and the changes in body weight (Figure 2B) were significantly lower in contrast with those of the Control group. From days 1 to 22, the occult blood (OB) test score (Figure 2C) gradually started to be different from that of the Control group, while disease activity index (DAI) scores (Figure 2D), which were significantly higher than those of the Control group, exhibited a similar trend to OB scores. In addition, the colonic weight (Figure 2G), index of colon length (Figure 2I), index of colonic weight (Figure 2J), spleen weight (Figure 2K), and the spleen weight index (Figure 2L) of mice in the DSS group statistically increased, whereas the body weight in the last day (Figure 2H) and colonic length (Figure 2E and F) decreased significantly. Meanwhile, the histopathological analysis (Figure 2M) showed that the colonic mucosa of mice was intact in the control group, with no obvious ulceration or inflammatory cell infiltration, neat arrangement of cup-shaped cells, and no congestion or edema, while the colitis mice showed exudation of the colonic mucosal epithelium, changes in crypt structure, disordered arrangement, local ulcer formation, hyperemia and edema in the colon mucosa, and infiltration of inflammatory cells in the lamina propria and submucosa, and the pathological injury scores were significantly increased (Figure 2N), which was consistent with our previous study. The above findings indicated that the chronic colitis model has been successfully constructed.

After Cur gavage administration, significant changes in body weight (Figure 2A), weight change rate (Figure 2B), OB scores (Figure 2C), and DAI scores (Figure 2D) were effectively reversed in colitis mice. Also, colon weight (Figure 2G), colon mass per unit length (Figure 2J), index of colonic weight (Figure 2I), spleen weight (Figure 2K), and spleen weight index (Figure 2L) were significantly decreased when compared with those in the DSS group, while the body weight in the last day (Figure 2H) and colonic length (Figure 2E and F) were obviously higher in the DSS + Cur group than in the DSS group. Meanwhile, ulceration and inflammatory infiltration in the colitis mice treated with Cur were distinctly improved (Figure 2M), and the pathological injury scores (Figure 2N) were observably decreased compared to those of colitis mice without treatment. Moreover, the above data were not evidently different between the Control and Control + Cur groups. Therefore, these data suggest that Cur can effectively alleviate DSS-induced colonic injury.

Cur regulates inflammatory cytokine expression

UC is a form of IBD characterized by rising levels of pro-inflammatory factors and declining levels of anti-inflammatory factors[22]. Disruption of the balance between pro-inflammatory and anti-inflammatory factors is one of the important characteristics of IBD[23].

In our experiment, we found that the levels of IL-6 (Figure 3C), IL-17A (Figure 3D), IL-1β (Figure 3E), and TNF-α (Figure 3F) were remarkably increased in the DSS group compared to the Control group, while the IL-10 (Figure 3A) and IL-35 levels (Figure 3B) in the DSS group were dramatically decreased, suggesting that the imbalance of pro-inflammatory and anti-inflammatory factors was one of the

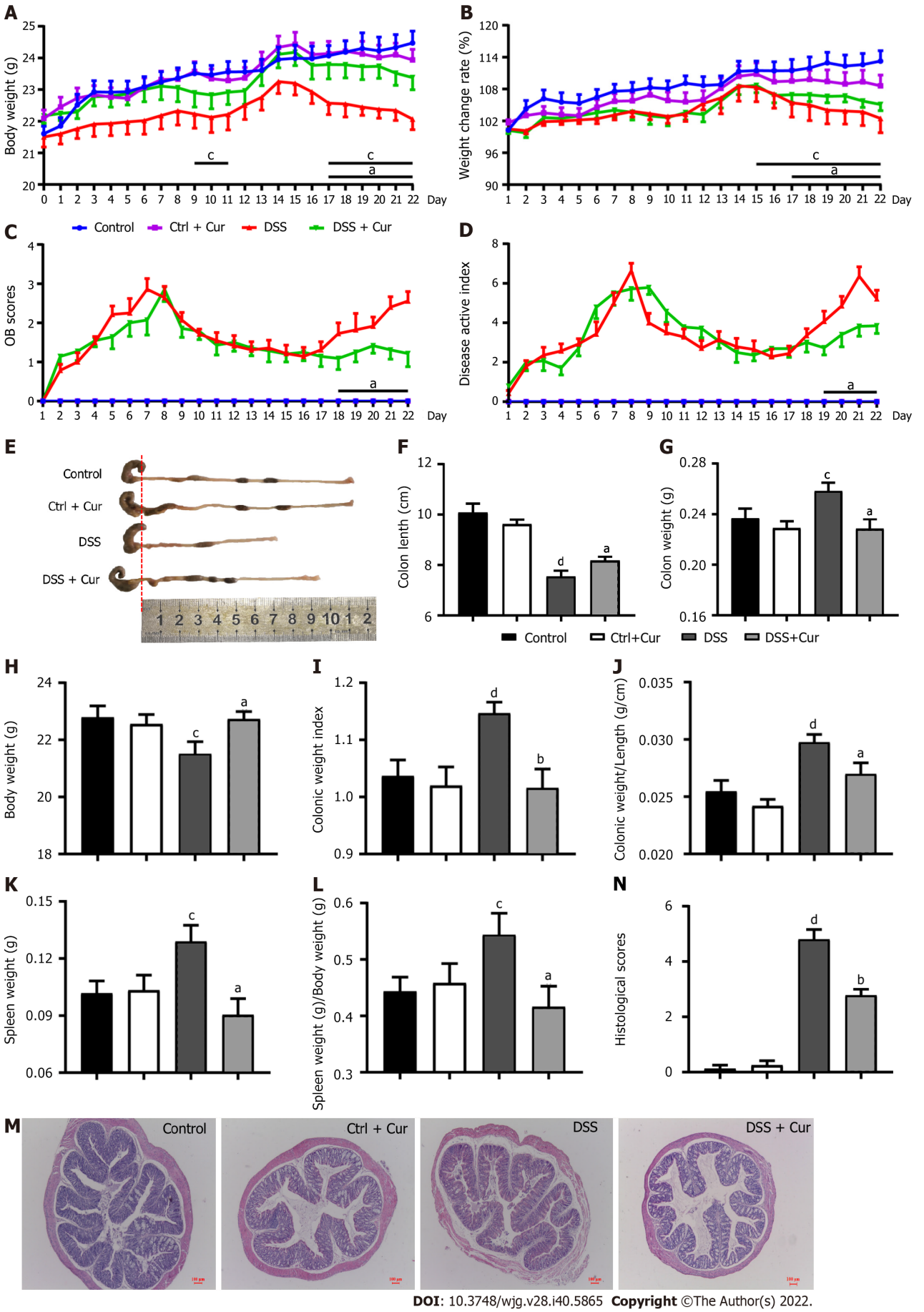

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Figure 2 Therapeutic evaluation of curcumin on DSS-induced experimental colitis. A: Body weight of mice from days 0-22; B: Weight change of rate

in mice from days 1-22; C: OB scores of mice in the four groups from days 1-22; D: DAI scores of mice in the four groups from days 1-22; E: Gross changes in colonic length; F: Colonic length of mice on day 22 in the four groups; G: Colonic weight; H: Body weight; I: Colonic weight index; J: Colonic weight/colonic length; K: Spleen weight; L: Spleen weight/body weight; M: Histological appearance of colons from individual groups of mice (hematoxylin and eosin staining, magnification 50 ×, scale bar = 100 μm); N: Pathological injury score. Data are presented as the mean ± SE (n = 8-10). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* DSS group.

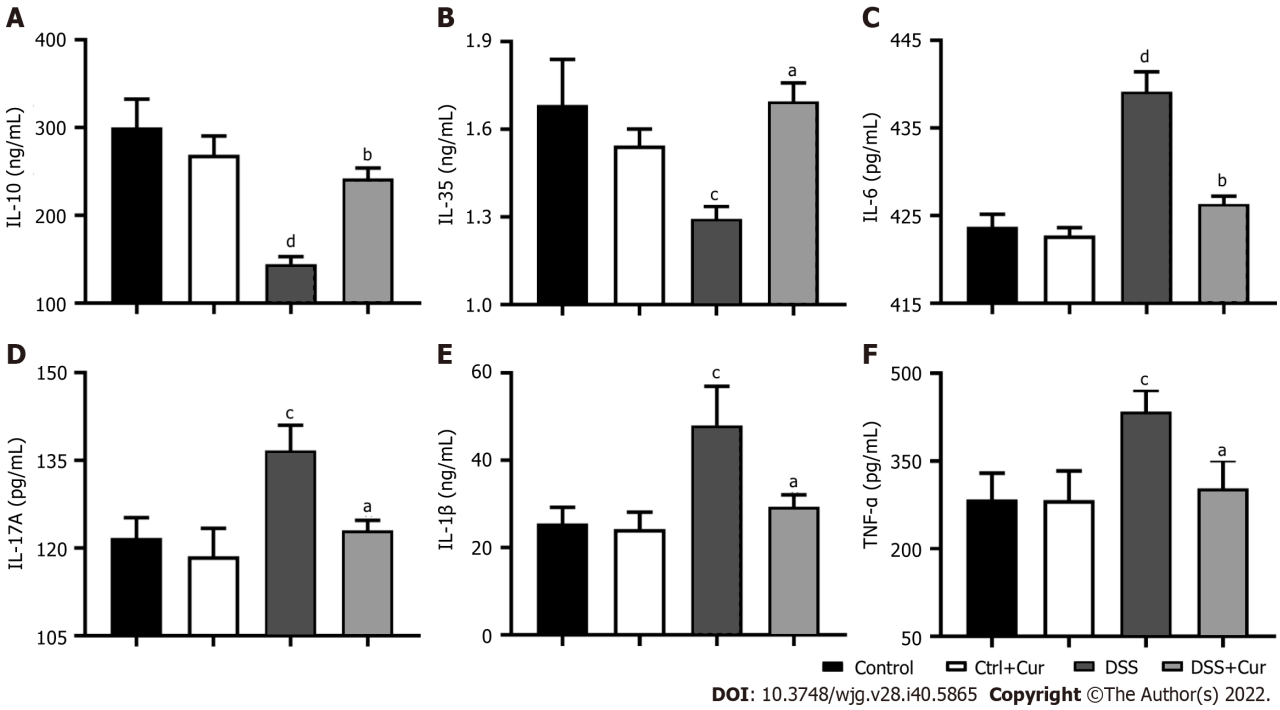

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Figure 3 Curcumin effectively regulates inflammatory cytokine expression. A: Concentration of IL-10 in colonic tissue; B: Concentration of IL-35 in colonic tissue; C: Concentration of IL-6 in colonic tissue; D: Concentration of IL-17A in colonic tissue; E: Concentration of IL-1β in colonic tissue; F: Concentration of TNF-α in colonic tissue. Data are presented as the mean ± SE (n = 8-10). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* DSS group.

important characters in DSS-induced colitis. More importantly, the IL-6 (Figure 3C), IL-17A (Figure 3D), IL-1β (Figure 3E), and TNF-α (Figure 3F) expression was significantly downregulated, while IL-10 (Figure 3A) and IL-35 (Figure 3B) expression was observably upregulated in colitis mice after Cur treatment. Altogether, Cur could effectively decrease IL-1β, IL-6, IL-17A and TNF-α production and increase IL-10 level, thus reestablishing the equilibrium between anti-inflammatory and pro-inflammatory factors in colitis.

Cur regulates memory B cell subsets

In the humoral immune response, there are two layers of memory cells, namely, long-lived B memory cells and B effector cells (plasma cells). The production of long-lived cells depends on the antigenic activation of B cells in the presence of T helper cells to induce germinal centers[24]. When the body comes in second contact with an antigen, memory B cells are quickly activated, differentiate into new tolerant or inducible plasma cells, secrete high-quality antibodies, and produce more efficient, faster, and more specific humoral immunity[9]. The tolerant type generally expresses factors such as IL-10 and TIM-3, while the induced type usually expresses molecules such as IgG, IgM, IgA, PD-1, CD38, CXCR3, FCRL5, CD103, and FasL, thus having opposite roles in the process of disease development[25]. Their balance determines the development, genesis, and development of memory B cells, while their dysfunction is one of the pathogenesis of IBD.

Recent studies have revealed that CD27 is expressed at higher levels in memory B cells than in plasma blasts and can be used as a biological biomarker of memory B cells. The CD19-positive cells were selected as the gate of B cells. Memory B cells ($CD19^+CD27^+$) could be marked and separated according to the expression of CD27 and CD19, which could then be divided into many subgroups in accordance with the expression of different cytokines on the surface of memory B cells[26,27].

In this study, compared with mice in the Control group, the level of $CD19^+CD27^+$ memory B cells in peripheral blood of colitis mice was significantly decreased (Figure 4A-D), the percentages of $CD19^+CD27^+IgM^+$ (Figure 5A and B), $CD19^+CD27^+IgG^+$ (Figure 5C and D), and CD19 + $CD27^+IgA^+$ (Figure 5E and F) cells were obviously increased, and the percentages of $CD19^+CD27^+FCRL5^+$ (Figure 6A and B),

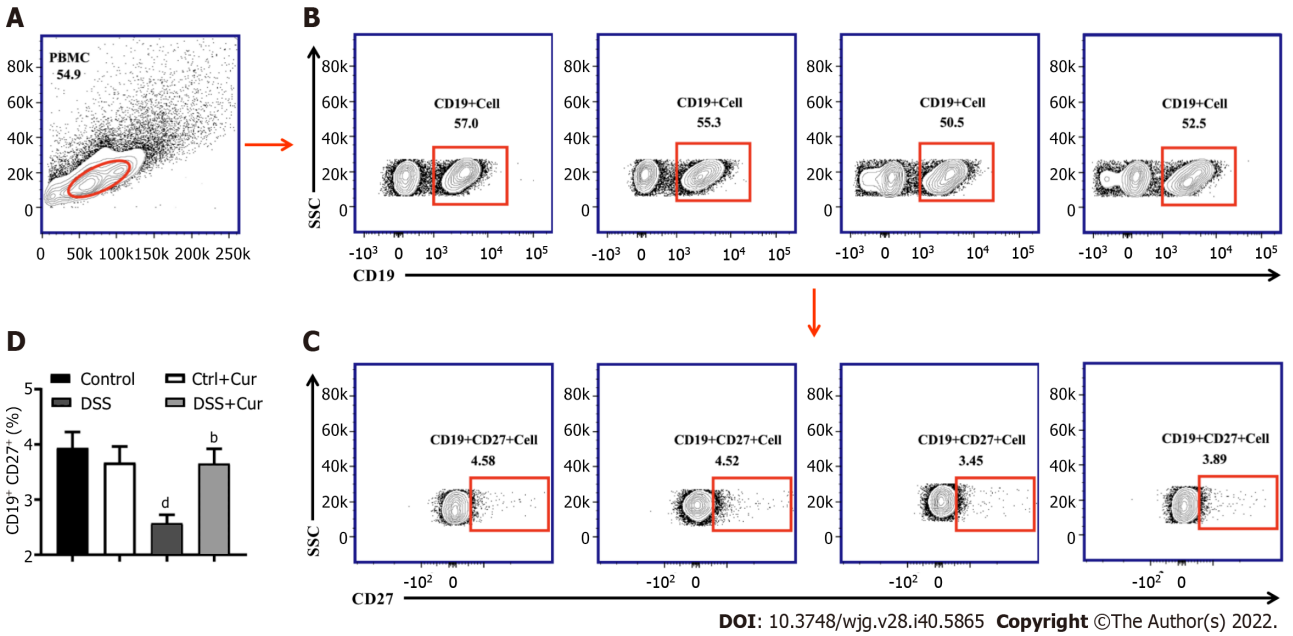

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Figure 4 Curcumin regulates differentiation of memory B cells in DSS-induced ulcerative colitis mice. A: Representative flow cytometry profile of peripheral blood mononuclear cells (PBMC); B: Representative flow cytometry profile of CD19$^+$ cells in peripheral blood; C: Representative flow cytometry profile of CD19$^+$ CD27$^+$ cells in peripheral blood; D: Bar chart of CD19$^+$ CD2$^+$ memory B cells. Data are presented as the mean ± SE (n = 8-10). $^aP < 0.05$, $^bP < 0.01$ *vs* Control group; $^cP < 0.05$, $^dP < 0.01$ *vs* DSS group.

CD19$^+$ CD27$^+$ CD103$^+$ (Figure 6C and D), CD19$^+$ CD27$^+$ FasL$^+$ (Figure 6G and H), CD19$^+$ CD27$^+$ PD-1$^+$ (Figure 7A and B), CD19$^+$ CD27$^+$ CD38$^+$ (Figure 7C and D), and CD19$^+$ CD27$^+$ CXCR3$^+$ (Figure 7E and F) cells were significantly increased. These percentages were significantly decreased after DSS-induced colitis mice were treated with Cur for 14 d. Meanwhile, the percentages of CD19$^+$ CD27$^+$ IL-10$^+$ (Figure 6E and F) and CD19$^+$ CD27$^+$ Tim-3$^+$ (Figure 7G and H) cells were remarkably decreased in mice of the DSS group, whereas the percentages of CD19$^+$ CD27$^+$ Tim-3$^+$ and CD19$^+$ CD27$^+$ IL-10$^+$ cells were markedly increased after experimental colitis mice were treated with Cur. These results suggested that Cur could effectively regulate memory B cell subsets in mice with colitis.

Cur regulates the activation of the Bcl-6-Syk-BLNK signaling pathway

Next, we used Western blot analysis to explore the effect of Cur on the regulation of memory B-cell-related signaling pathways in mice with colitis. The Bcl-6-Syk-BLNK signaling pathway is a crucial signaling pathway involved in the activation, differentiation, proliferation, and functional display of immune cells, including B cells and memory B cells. In our experiments, the expression levels of Bcl-6 (Figure 8A and B), CIN85 (Figure 8A and E), Syk (Figure 8A and F), and p-Syk (Figure 8A and G) were higher in the DSS group than in the Control group. After 14 d of Cur treatment, Bcl-6, CIN85, Syk, and p-Syk expression in the colonic tissues of colitis mice was significantly inhibited. In addition, the expression levels of BLNK (Figure 8A and C) and p-BLNK (Figure 8A and D) were obviously lower in the DSS group than in the Control group, and the expression levels of BLNK and p-BLNK in the treatment group were evidently increased compared with the DSS group. Thus, these results suggested that Cur effectively regulates the activation of the Bcl-6-Syk-BLNK signaling pathway in colitic mice.

DISCUSSION

The incidence of UC has been gradually increasing worldwide, especially in adults between 30 and 40 years old. Patients with UC have mucosal inflammation, which starts in the rectum but continues into the proximal colon, usually presenting with bloody stool. Its pathogenesis is multifactorial, involving destroyed epithelial barrier, dysregulated immune responses, genetic predisposition, and imbalance of the gut microbiota. The dysfunction of immune cells and the destruction of immune homeostasis are deemed as the direct causes of UC[28,29].

B cells have an important role in adaptive immunity, and can differentiate into plasma cells and participate in inflammatory responses by producing a large number of antibodies and suppressing immune responses by releasing anti-inflammatory cytokines[30]. Previous studies on the role of B cells in immune diseases have indicated that the disruption of B cell subsets was a common characteristic in various autoimmune diseases. The variations in B cell frequency, abnormal molecular expression, key

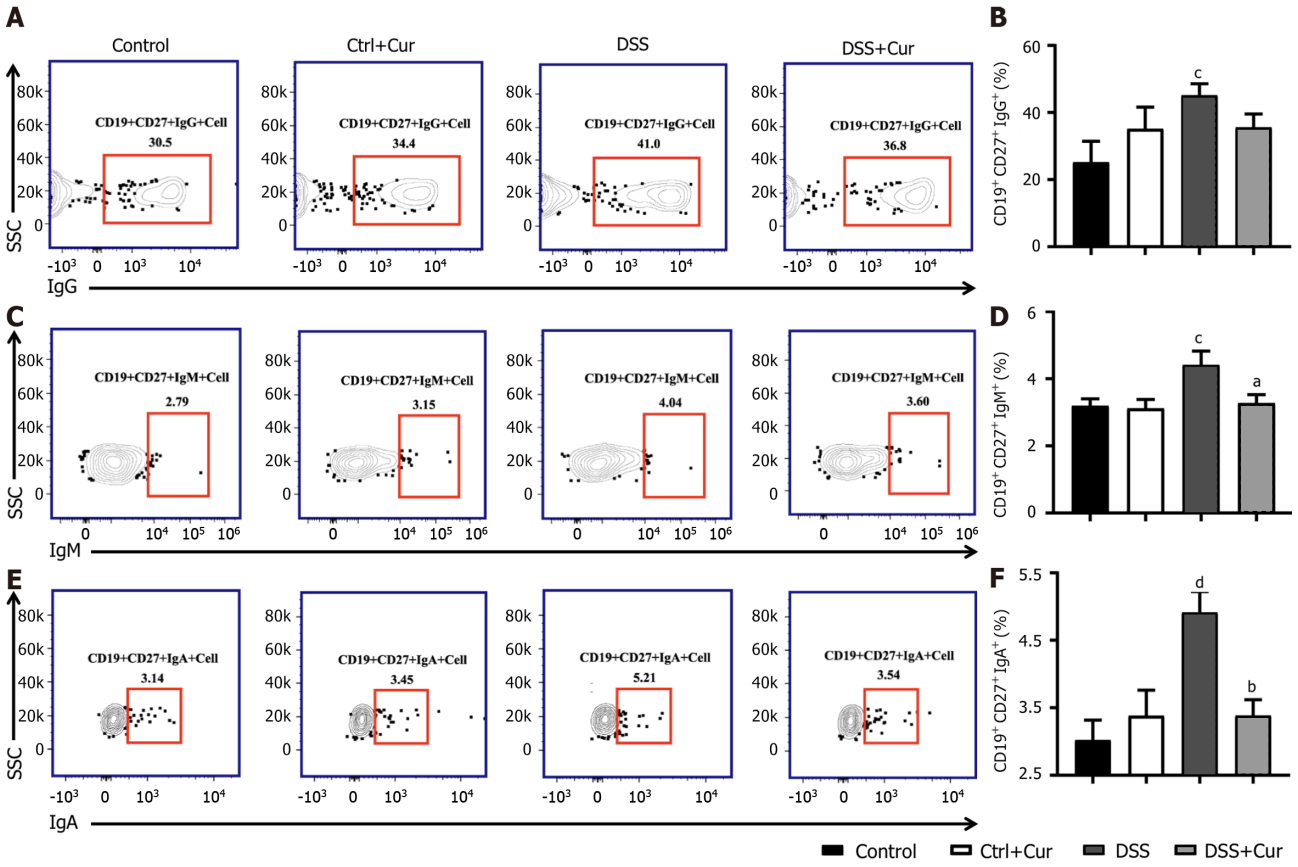

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Figure 5 Curcumin regulates differentiation of memory B cells in DSS-induced ulcerative colitis mice. A: Representative flow cytometry profile of $CD19^+$ $CD27^+$ IgG^+ cells in peripheral blood; B: Bar chart of $CD19^+$ $CD27^+$ IgG^+ cells; C: Representative flow cytometry profile of $CD19^+$ $CD27^+$ IgM^+ cells in peripheral blood; D: Bar chart of $CD19^+$ $CD27^+$ IgM^+ cells; E: Representative flow cytometry profile of $CD19^+$ $CD27^+$ IgA^+ cells in peripheral blood; F: Bar chart of $CD19^+$ $CD27^+$ IgA^+ cells. Data are presented as the mean ± SE (n = 8-10). $^aP < 0.05$, $^bP < 0.01$ *vs* Control group; $^cP < 0.05$, $^dP < 0.01$ *vs* DSS group.

pathways, and the imbalance in subgroup distribution are closely related to the immunological pathogenesis of many diseases. Different B cell subsets can promote and inhibit inflammation. Overactivation of B cell responses, abnormal expression of signaling factors and cytokines, and imbalance in subpopulations, including memory B cells, are involved in the pathogenesis of many immune diseases. Meanwhile, memory B cells were considered as the source of several pro-inflammatory cytokines, which are involved in the pathogenesis of many autoimmune diseases[31]. In recent years, domestic and foreign scholars have extensively focused on this special class of B cells and their relationship with autoimmune diseases, such as myasthenia grais, Gillan barre syndrome, and other immune system diseases[32]. Dysregulation of memory B cells was found in several autoimmune diseases. Previous studies[17] have found that the B cell immune response, namely, the antibody response, is abnormally activated, and the memory B cells are significantly reduced in UC patients.

It is known that the body's immune system generates memory during the body's first antigen-antagonism and antibody response to specific antigens. The initial B cells can transform into memory B cells to complete the rapid category conversion in the next immunization[33]. These plasma cells migrate to bone marrow and lymphoid tissue, secrete specific antibodies such as immunoglobulins, inflammatory cytokines, and chemokines, mediate rapid and effective secondary immune response, and have an important role in humoral immunity[34].

When memory B cells are stimulated again by the same antigen, they are rapidly activated to produce IgG, IgM, IgA, FCRL5, CD103, IL-10, FasL, PD-1, CD38, CXCR3, Tim3, and so on. It is known that the destruction of the intestinal mucosal barrier during the onset of IBD causes abnormal expression of immunoglobulins, which are a major participant in humoral immunity, including IgG, IgA, IgM, *etc* [35]. IgG has the function of opsonizing phagocytes and neutralizing toxins, IgA has an antibacterial and antiviral function, while IgM has the function of lysing pathogens and binding antigens. Previous studies have shown that the increased number of endoplasmic cells in the intestinal mucosa of IBD patients leads to an increase in the secretion of IgG, IgA, IgM, and other immunoglobulins, which further participate in the formation of local immune complexes in the intestinal mucosa and trigger intestinal inflammation[36]. "FCR-like" (FCRL) is an evolutionarily conserved gene family associated with IgG and IgE Fc receptors (FCRS). FCRL5 is preferentially expressed in B cells and encodes

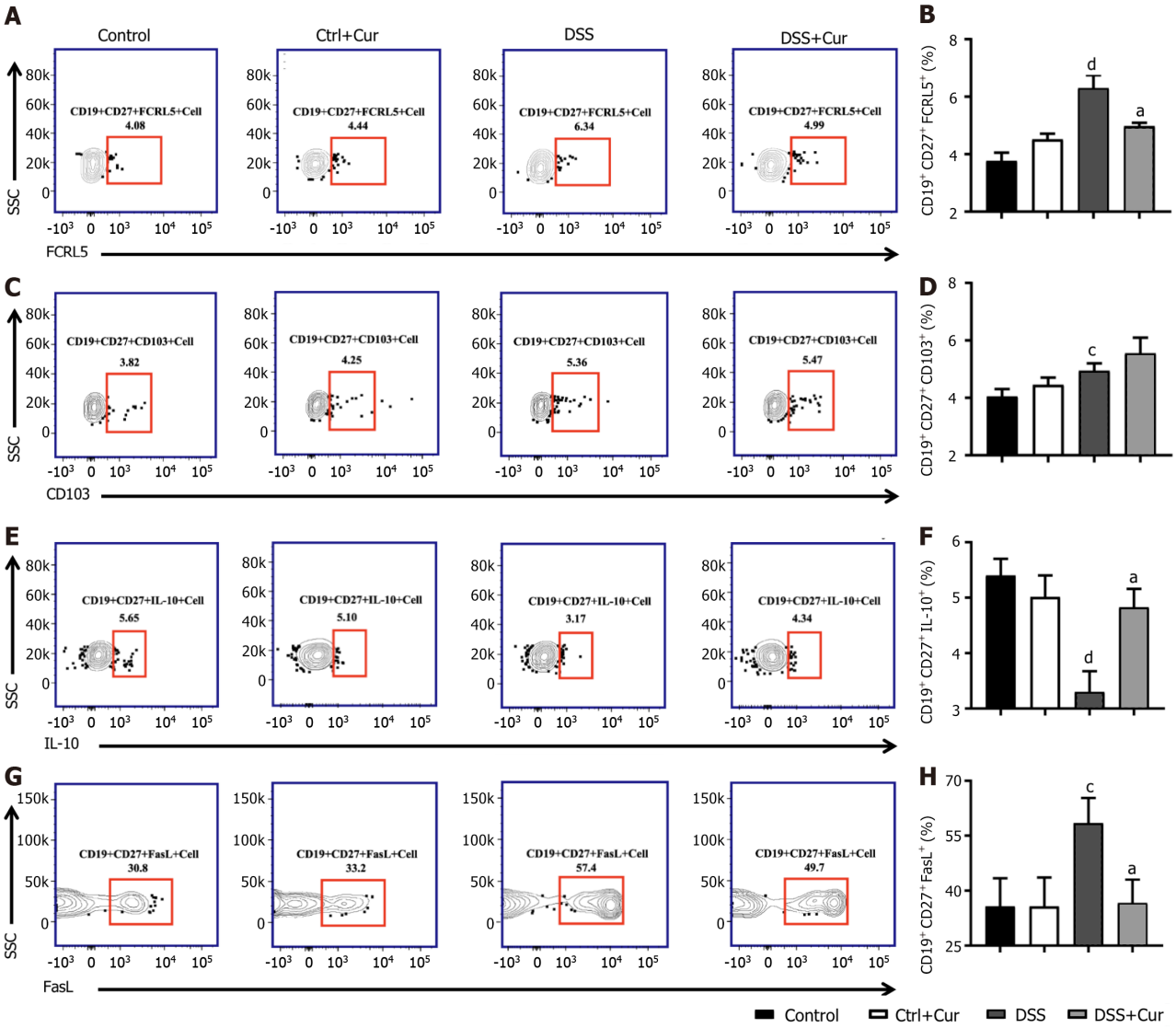

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Figure 6 Curcumin regulates differentiation of memory B cells in DSS-induced ulcerative colitis mice. A: Representative flow cytometry profile of $CD19^+CD27^+FCRL5^+$ cells in peripheral blood; B: Bar chart of $CD19^+CD27^+FCRL5^+$ cells; C: Representative flow cytometry profile of $CD19^+CD27^+CD103^+$ cells in peripheral blood; D: Bar chart of $CD19^+CD27^+CD103^+$ cell; E: Representative flow cytometry profile of $CD19^+CD27^+IL\text{-}10^+$ cells in peripheral blood; F: Bar chart of $CD19^+CD27^+IL\text{-}10^+$ cells; G: Representative flow cytometry profile of $CD19^+CD27^+FasL^+$ cells in peripheral blood; H: Bar chart of $CD19^+CD27^+FasL^+$ cells. Data are presented as the mean ± SE (n = 8-10). $^aP < 0.05$, $^bP < 0.01$ *vs* Control group; $^cP < 0.05$, $^dP < 0.01$ *vs* DSS group.

transmembrane proteins with tyrosine-based immunoregulatory motifs[37]. CD103 (human mucosal lymphocyte antigen-1) is highly expressed at mucosal sites, and CD103-positive lymphocytes have a remarkable ability to move directly toward the intestinal epithelial cell model and immobilize mucosal lymphocytes with E-cadherin at the basolateral side of the intestinal epithelium[38]. IL-10 has anti-inflammatory effects by inhibiting inflammation and cellular immune response, improving B cell survival rate, B cell proliferation, MHC II antigen expression, and immunoglobulin secretion, and inhibiting the production of NK cytokines, monocytes, and macrophage pro-inflammatory factors[39]. In the gastrointestinal tract, IL-10 is secreted by T cells, B cells, macrophages, neutrophils, and natural killer cells, and maintains mucosal homeostasis and immune tolerance during inflammation[40]. FasL is a membrane receptor protein that belongs to the superfamily of nerve growth factor receptors. It has a signal transduction role in cell apoptosis, and can induce colon epithelial cells and protect Th2 cells from apoptosis or even directly kill Th2 cells. In colonic epithelial cells, FasL can mediate the apoptosis of colonic epithelial cells through caspase-mediated apoptosis, which ultimately leads to the destruction of the colonic mucosal barrier and the formation of ulcers[41]. Indeed, expressed of Fas L in B cells is upregulated by IL-10 in some autoimmune diseases and GVHD models. CD5 is expressed in both $FasL^+$ B cells and IL-10-producing B10 cells, suggesting the possibility of a feedforward cycle in which B cells can express IL-10 to enhance their other immunomodulatory mechanisms[42]. Programmed death receptor 1 (PD-1) is a coinhibitory receptor expressed on T cells, B cells, natural killer cells, and monocytes. It has been confirmed that PD-1 is highly expressed in intestinal mucosal cells. Its ligand

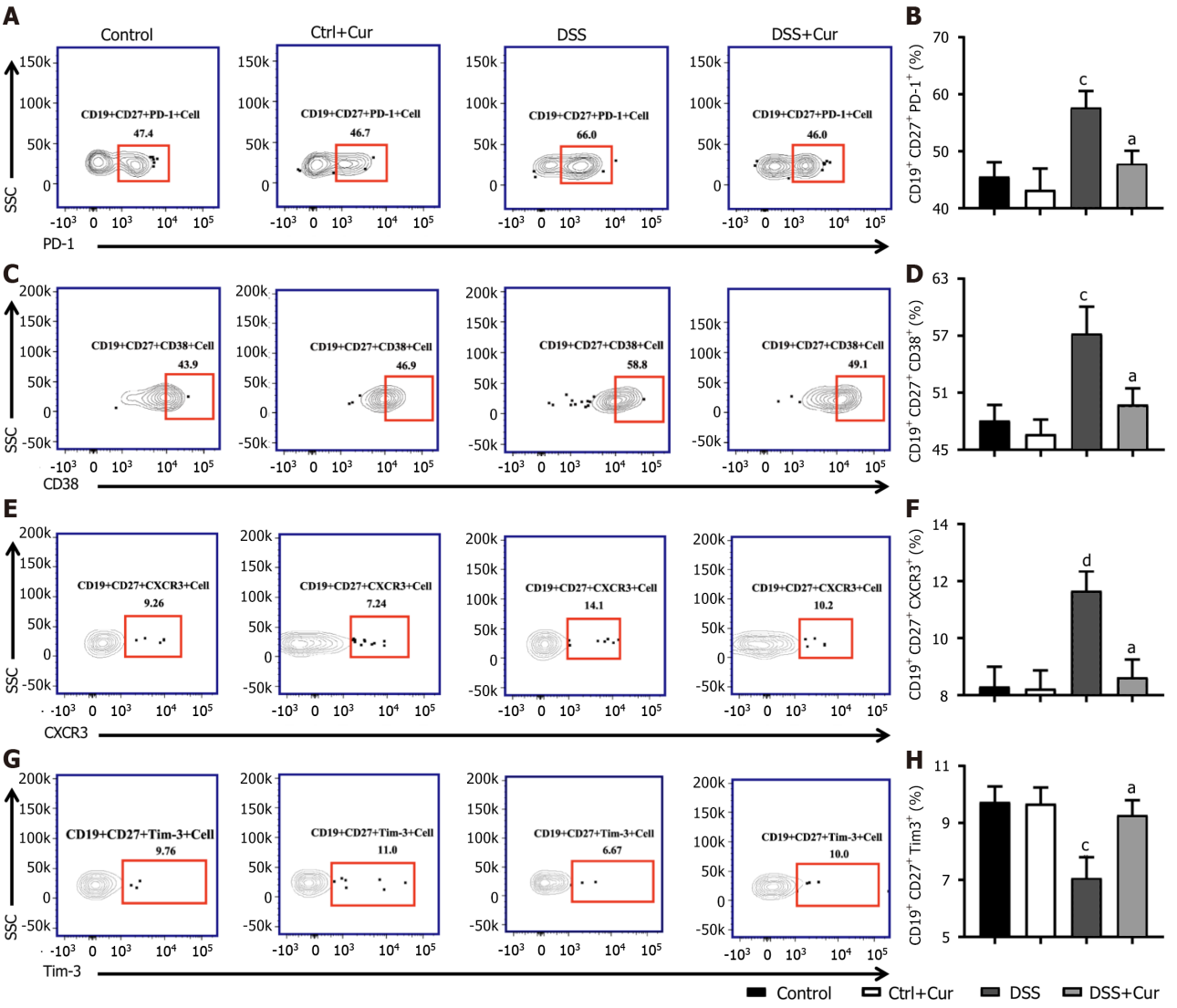

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Figure 7 Curcumin regulates differentiation of memory B cells in DSS-induced ulcerative colitis mice. A: Representative flow cytometry profile of $CD19^+CD27^+$ $PD\text{-}1^+$ cells in peripheral blood; B: Bar chart of $CD19^+CD27^+$ $PD\text{-}1^+$ cells; C: Representative flow cytometry profile of $CD19^+CD27^+$ $CD38^+$ cells in peripheral blood; D: Bar chart of $CD19^+CD27^+CD38^+$ cells; E: Representative flow cytometry profile of $CD19^+CD27^+CXCR3^+$ cells in peripheral blood; F: Bar chart of $CD19^+CD27^+CXCR3^+$ cells; G: Representative flow cytometry profile of $CD19^+CD27^+$ $Tim\text{-}3^+$ cells in peripheral blood; H: Bar chart of $CD19^+CD27^+$ $Tim\text{-}3^+$ cells. Data are presented as the mean ± SE (n = 8-10). $^aP < 0.05$, $^bP < 0.01$ *vs* Control group; $^cP < 0.05$, $^dP < 0.01$ *vs* DSS group.

binding can participate in the occurrence of intestinal mucositis by activating initial T lymphocytes, inhibiting activated effector T lymphocytes, and regulating the secretion of cytokines[43]. Studies have shown that in patients with UC, the expression level of PD-1 in inflammatory sites is significantly increased, while intestinal mucositis is alleviated when the PD-1 pathway is blocked[44]. CD38 is expressed on intestinal inflammatory cells, which has been reported to promote intestinal inflammation. Therefore, CD38 indirectly promotes intestinal inflammation[45]. CXCR3 is a chemokine receptor of the CXC family, expressed in epithelial and endothelial cells, as well as various lymphocytes such as NK cells, B cells, memory T cells, monocytes, and neutrophils. In IBD patients, CXCR3 and its corresponding ligands are strongly expressed in the intestinal mucosa. They can also recruit pro-inflammatory cells into the colon during colitis, leading to IBD[46]. As an important immune checkpoint molecule, Tim-3 regulates immune response. According to previous studies, Tim-3 is also expressed on the surface of B cells, while B cell activation affects the expression of Tim-3. After knocking out Tim-3, its protective effect disappeared, and the protective effect of B cells depended on Tim-3. Wang *et al*[47] found that during the development of acute enteritis, the expression of Tim-3 on the surface of infiltrated immune cells, especially B cells in the lamina propria of the colon, significantly decreased. After knocking out Tim-3, enteritis was significantly aggravated, and the protective effect of B cells on enteritis potentially depended on Tim-3. When the humoral immune balance related to memory B cells is disrupted, the expression of proinflammatory factors is induced, and the expression of anti-inflammatory factors is inhibited, leading to a variety of autoimmune diseases, including IBD[48].

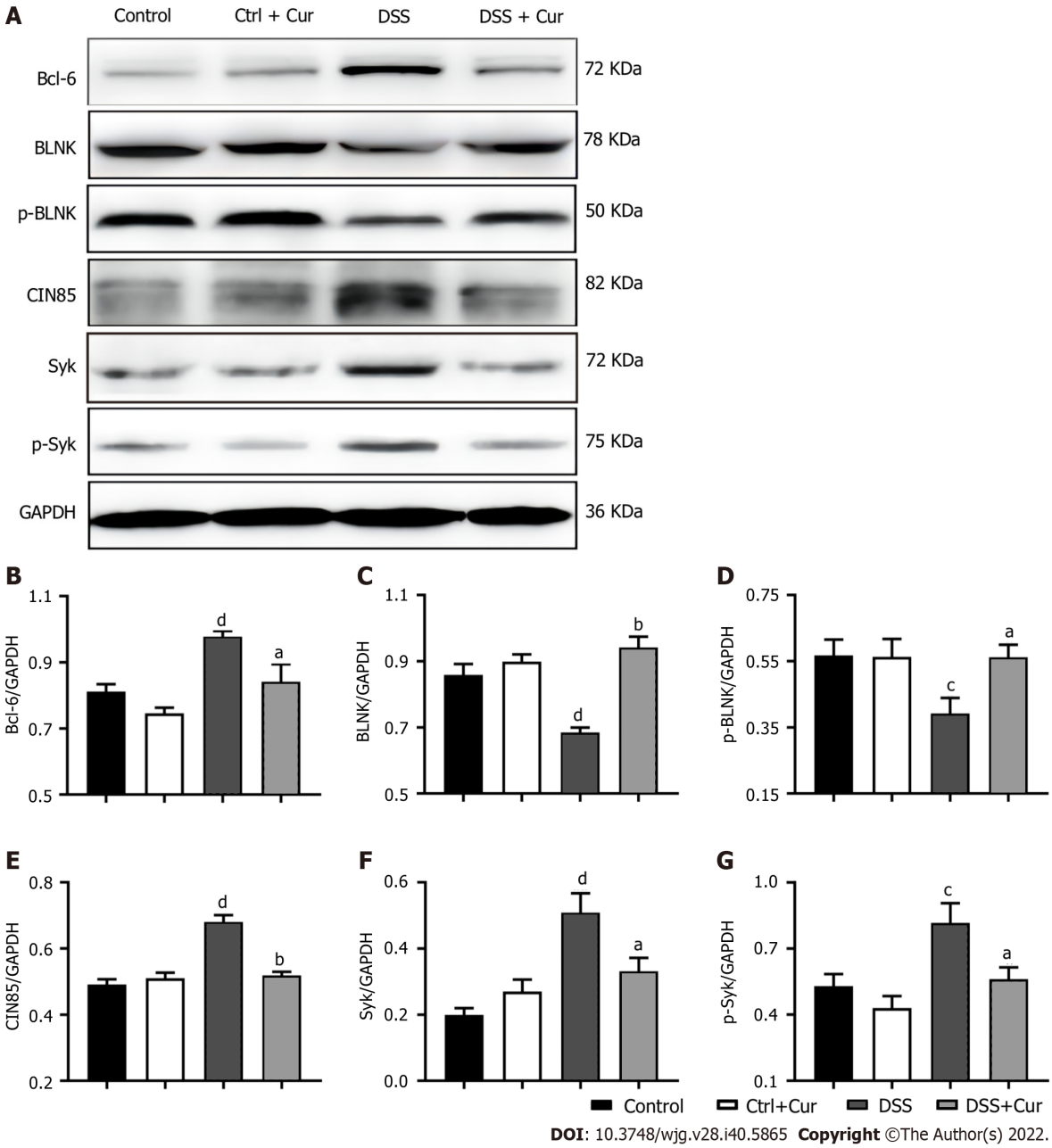

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Figure 8 Curcumin regulates expression of memory B cell-related proteins in DSS-induced ulcerative colitis mice. A: Expression of Bcl-6, BLNK, p-BLNK, CIN85, Syk, and p-Syk in colon tissues as revealed by Western blot analysis. GAPDH served as the internal reference; B-G: Quantitative evaluation of (B) Bcl-6, (C) BLNK, (D) p-BLNK, (E) CIN85, (F) Syk, and (G) p-Syk. Data are presented as the mean ± SE (n = 8-10). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* DSS group.

Our experimental results revealed that the level of memory B cells in the peripheral blood of DSS-induced colitis mice was obviously abnormal, which was consistent with other literature reports. In our experiment, induced memory B cells (IgG^+, IgM^+, IgA^+, $FCRL5^+$, $CD103^+$, $FasL^+$, $PD\text{-}1^+$, $CD38^+$, and $CXCR3^+$ memory B cells) were over-expressed and tolerant memory B cells ($IL\text{-}10^+$ and $Tim\text{-}3^+$ memory B cell) were under-expressed. After Cur administration, the levels of induced memory B cells with pro-inflammatory factor expression were significantly inhibited, while the levels of tolerant memory B cells and anti-inflammatory factors were increased. These phenomena were accompanied by Cur's effective inhibition of pathological damage in colitis mice, which indicated that Cur could effectively regulate the balance of memory B cells in colitis mice, so as to restore the balance of pro-inflammatory factors and anti-inflammatory factors and alleviate the symptoms of DSS-induced colitis. These findings suggest that the therapeutic effect of Cur on UC in mice might be closely related to the balance regulation of memory B cells.

In order to explore the mechanisms by which Cur regulates the balance of memory B cells, we examined the activation of the Bcl-6-Syk-BLNK signaling pathway in colon tissue. Bcl-6 protein is highly expressed in germinal B cells, and the germinal response is the maturation of antigen-specific B cells

into memory B cells, which constitute the cellular components of immune memory of the B cell lines [49]. Previous studies have suggested that Bcl-6 expression is necessary to enable germinal center B cells to differentiate into memory B cells. Bcl-6 expression was also found to enhance B cell survival and thereby regulate self-renewal of memory B cell. High expression of Bcl-6 sustains a germinal center B cell phenotype and inhibits the differentiation of memory B cells *in vitro*. Furthermore, it has been established that Syk/p-Syk is a key signal pathway, which is required to couple the BCR to all downstream signaling pathways in mature B cells. Also, the B cells with Syk deficiency could not differentiate into the germinal center or plasma cells, suggesting that Syk is required for the survival of memory B cells[50,51]. When BLNK/p-BLNK is deficient, the transformation of primordial B cells into memory B cells is disrupted, and the level of memory B cells is significantly reduced[52]. CIN85 is widely involved in various physiological activities in the body, especially vesicle transport, membrane transport, and invasion and migration of various cancer cells. It also participates in cell differentiation, cell cycle, and apoptosis[53,54]. According to the above analysis, the Bcl-6-Syk-BLNK signaling pathway plays a crucial role in the development, activation, and differentiation of B cells and memory B cells.

In our experiment, after Cur administration, the levels of other proteins were increased, but BLNK and p-BLNK protein expression was decreased. This result indicated that Cur could significantly inhibit the activation of the Bcl-6-Syk-BLNK signaling pathway, which is consistent with its regulatory effect on memory B-cell balance.

As a new member of regulatory immunity, the biological characteristics and functions of memory B cells have been studied extensively, providing a new perspective for studying immune regulation mode and a new method for treating IBD. It has been proven that Cur is effective against DSS-induced micron colitis. In this experiment, we found that Cur could effectively improve the mucosal damage of DSS-induced colitis mice and significantly regulate the balance of induced type and tolerance memory B cells in colitis mice. As a result, the Bcl-6-Syk-BLNK signaling pathway was significantly inhibited. The effectiveness of Cur for murine UC was closely related to the regulation of memory B cell balance. Cur regulated the balance of memory B cell subgroups in mice with colitis, which was realized by activating the Bcl-6-Syk-BLNK signaling pathway. These results suggest that through the activation of the Bcl-6-Syk-BLNK signaling pathway, Cur harmonizes the differentiation and activation of inducible and inhibitory memory B cells, promotes the balance between them, reduces the expression of chemokines related to the inhibition of inducible memory B cells, and prevents excessive accumulation of inflammatory cells. It can also prevent overproduction of pro-inflammatory factors, increase the expression of tolerant memory B cells and anti-inflammatory factors to alleviate the immune damage by correcting memory B cell disorder, and maintain the humoral immune homeostasis mediated by memory B cells.

CONCLUSION

Cur could effectively alleviate DSS-induced colitis in mice by regulating memory B cells and the Bcl-6-Syk-BLNK signaling pathway.

ARTICLE HIGHLIGHTS

Research background

Curcumin (Cur) has shown promising efficacy in experimental colitis mice and ulcerative colitis patients. Disturbance in memory B cells has been observed in various autoimmune diseases, including inflammatory bowel disease. However, few studies have explored whether Cur treatment of colitis is associated with memory B cells.

Research motivation

To the best of our knowledge, this is the first study to explore the mechanisms by which Cur regulates memory B cells in the treatment of experimental colitis.

Research objectives

To investigate whether Cur can alleviate experimental colitis induced by DSS through regulating memory B cells and Bcl-6-Syk-BLNK signaling.

Research methods

Cur (100 mg/kg/d) was intragastrically administered in mice with colitis induced by DSS for 14 consecutive days. The effect of Cur was evaluated by macroscopic and histological observation. The levels of memory B cell subgroups in mouse peripheral blood were detected by flow cytometry, and the levels of cytokines in colonic tissue homogenates were measured by using ELISA. The expression of Bcl-6, BLNK, and Syk was measured by Western blot analysis.

Research results

After mice were treated with Cur for 14 d, the body weight, colonic weight and length, colonic weight index, and histopathological injury were ameliorated. In colitis mice, the concentrations of IL-1β, IL-6, TNF-α, and IL-7A were significantly decreased, while the concentrations of anti-inflammatory cytokines IL-35 and IL-10 were obviously increased. Activation of memory B cell subsets in colitis mice was confirmed by a remarkable reduction in the expression of IgM^+, IgG^+, IgA^+, $FCRL5^+$, $CD103^+$, $FasL^+$, $PD\text{-}1^+$, $CD38^+$, and $CXCR3^+$ on the surface of $CD19^+CD27^+$B cells, while the number of $CD19^+$ $CD27^+$ $IL\text{-}10^+$ and $CD19^+CD27^+$ $Tim\text{-}3^+$ B cells increased significantly. In addition, Cur observably decreased the protein levels of Syk, p-Syk, Bcl-6, and CIN85, and increased BLNK and p-BLNK expression in colitis mice.

Research conclusions

Cur could effectively alleviate DSS-induced colitis in mice, which is realized *via* a potential mechanism involving memory B cells and the Bcl-6-Syk-BLNK signaling pathway.

Research perspectives

In the present study, Cur effectively ameliorated the pathological colonic injury induced by DSS, which was achieved through a potential mechanism involving regulating the balance of memory B cells and activating the Bcl-6-Syk-BLNK signaling pathway.

FOOTNOTES

Author contributions: Wei SY and Wu TT contributed equally to this work and should be regarded as co-first authors; Huang JQ, Zhong YB, Kang ZP, Wang MX, Zhou BG, Zhao HM, and Ge W performed the experiments; Liu DY and Wang HY contributed reagents/materials/analytical tools; Liu DY and Zhong YB analyzed the data; Wei SY, Wu TT, and Liu DY wrote the paper; Liu DY and Wang HY conceived and designed the experiments.

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ORIGINAL ARTICLE

Observational Study

Liver transplantation is beneficial regardless of cirrhosis stage or acute-on-chronic liver failure grade: A single-center experience

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Abstract

BACKGROUND

Liver transplantation for the most critically ill remains controversial; however, it is currently the only curative treatment option.

AIM

To assess immediate posttransplant outcomes and compare the short (1 year) and long-term (6 years) posttransplant survival among cirrhotic patients stratified by disease severity.

METHODS

We included cirrhotic patients undergoing liver transplantation between 2015 and 2019 and categorized them into compensated cirrhosis (CC), decompensated cirrhosis (DC), and acute-on-chronic liver failure (ACLF). ACLF was further divided into severity grades. Our primary outcomes of interest were total days of intensive care unit (ICU) and hospital stay, development of complications and posttransplant survival at 1 and 6 years.

RESULTS

235 patients underwent liver transplantation (CC = 11, DC = 129 and ACLF = 95). Patients with ACLF had a significantly longer hospital stay [8.0 (6.0-13.0) *vs* CC, 6.0 (3.0-7.0), and DC 7.0 (4.5-10.0); $P = 0.01$] and developed more infection-related complications [47 (49.5%), *vs* CC, 1 (9.1%) and DC, 38 (29.5%); $P < 0.01$]. Posttransplant survival at 1- and 6-years was similar among groups ($P = 0.60$ and $P = 0.90$, respectively). ACLF patients stratified according to ACLF grade [ACLF-1 $n = 40$ (42.1%), ACLF-2 $n = 33$ (34.7%) and ACLF-3 $n = 22$ (23.2%)], had similar ICU and hospital stay length ($P = 0.68$, $P = 0.54$), as well as comparable frequencies of overall and infectious post-transplant complications ($P = 0.58$, $P = 0.80$). There was no survival difference between ACLF grades at 1 year and 6 years ($P = 0.40$ and $P = 0.15$).

CONCLUSION

Patients may benefit from liver transplantation regardless of the cirrhosis stage. ACLF patients have a longer hospital stay and frequency of infectious complications; however, excellent, and comparable 1 and 6-year survival rates support their enlisting and transplantation including those with ACLF-3.

Key Words: Liver transplantation; Acute-on-chronic liver failure; Prognosis; Survival analysis; Critical care

Core Tip: Cirrhotic patients classified into compensated or decompensated cirrhosis and acute-on-chronic liver failure (ACLF) underwent liver transplantation. Patients with ACLF have a longer hospital stay and a higher frequency of infectious complications, but despite that, have similar posttransplant survival at one year and up to 6 years of follow-up.

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INTRODUCTION

Cirrhosis is the consequence of chronic liver disease originated by a variety of etiological factors, characterized by the disruption of the normal hepatic architecture due to fibrosis with consequent hemodynamic repercussions. Unless the hepatic insult is removed, patients with this condition will suffer progression and transition from a stage of compensated cirrhosis (CC) to a stage of decompensated cirrhosis (DC) with the occurrence of portal hypertension-related symptoms[1]. An entity of recent definition known as acute-on-chronic liver failure (ACLF) is now recognized[2,3], which imposes the highest mortality risk given by a state of profound cirrhosis-associated immune dysfunction and the development of organ failures additional to that of the liver[4,5].

Currently, liver transplantation (LT) is the only definitive therapeutic measure for any of these patients, albeit with the implied risks including posttransplant complications and the long-term use of immunosuppressive drugs. However, patients benefit in general from excellent posttransplant survival.

There is controversial literature, and uncertainty prevails concerning the possible futility of assigning a liver to a patient with advanced cirrhosis and systemic alterations such as in those with ACLF. Some studies have demonstrated the presence of ACLF at the time of LT as a risk factor for mortality and graft loss, and that these patients may have lower short and long-term survival after transplant[6-8]. However, others have shown non-significant survival differences between ACLF and non-ACLF patients including a marked improvement in the prognosis of those with the highest severity (ACLF-3) [9-12]. The development of early allograft dysfunction and renal dysfunction is also comparable between these groups, as well as long-term liver and kidney function[12,13]. However, unfavorable outcomes can be expected and a higher frequency of perioperative and postoperative complications has been reported[8,9]. Differences may be found when ACLF patients are subdivided by ACLF grade, however, survival disparities are still controversial[7,9].

We here report our transplant center's experience in an effort to further contribute to the evidence on the benefit of LT in ACLF. The aim was to assess immediate posttransplant outcomes and to compare the short (1 year) and long-term (6 years) posttransplant survival among cirrhotic patients stratified by

disease severity. Unlike other studies so far, this study specifically compares survival and outcomes between compensated, decompensated cirrhosis and ACLF, thus distinctly contrasting the extremes of disease severity. Additional analyses were performed to determine possible differences between ACLF grades. These results should encourage further transplantation in those with this severe form of cirrhosis and even in patients with ACLF grade 3.

MATERIALS AND METHODS

Patients and operational definitions

This study included all patients undergoing LT between January 1st 2015 and December 31st 2019. Patients with a previous transplant, malignancies other than hepatocellular carcinoma, fulminant hepatic failure, and amyloidosis were excluded. Patients were classified into compensated cirrhosis (CC), decompensated cirrhosis (DC), and ACLF, and the latter were further subdivided into ACLF grades 1, 2 and 3 (Figure 1). Diagnoses of CC and DC were based on the absence or presence of symptoms related to portal hypertension, including ascites, encephalopathy, or variceal bleeding, respectively, as previously described[14]. All CC patients received liver transplant because of hepatocarcinoma mainly due to hepatitis C virus (HCV) infection.

ACLF was diagnosed in a patient that fulfilled ACLF criteria any time during their clinical course while waiting to receive a LT according to the EASL-CLIF consortium criteria[2] which state the following organ failure (OF) definitions: liver (total bilirubin ≥ 12 mg/dL); kidney (creatinine ≥ 2 mg/dL); brain (encephalopathy grade 3 or 4 according to West-Haven criteria); coagulation (INR ≥ 2.5); circulation (vasopressor use due to circulatory failure); and lung ($PaO_2/FiO_2 \leq 200$ or $SpO_2/FiO_2 \leq 214$ or mechanical ventilation due to lung failure). ACLF grading was performed as follows: ACLF-1, patients with single kidney OF or non-renal OF plus kidney dysfunction (creatinine between 1.5-1.9 mg/dL) and/or brain dysfunction (encephalopathy grade 1 or 2 according to West-Haven criteria); ACLF-2, patients with two OFs; and ACLF-3, patients with three or more OFs.

Patients at our center are considered for LT based on cirrhosis disease severity according to an unrestricted evaluation of the Model for End-Stage Liver Disease (MELD), MELD-Na and CLIF-C scores and are ultimately listed according to an interdisciplinary consensus reached by the gastroenterology, cardiology, pneumology, infectology, otorhinolaryngology, psychiatry, surgery, anesthesiology, and stomatology specialties. Liver transplants were carried out in their majority with classic technique. Briefly, recipient hepatectomy involved a bilateral subcostal incision with or without midline extension. Then dissection and clamping of the portal vein, hepatic artery, bile duct, and superior and inferior vena cava were done. Implantation of the donor's liver was attained by anastomosing first the superior vena cava from the donor with that of the recipient, followed by the inferior vena cava, and portal vein, after which reperfusion of the donor liver was begun. Total reperfusion was then obtained by anastomosing the hepatic artery of the graft with the junction of the gastroduodenal artery and the common hepatic artery of the recipient. The procedure was completed after performing cholecystectomy and duct to-duct anastomosis.

The immunosuppressive regimen in all patients following the procedure consisted of all or a combination of the following drugs: a calcineurin inhibitor (tacrolimus or cyclosporine), corticosteroids, mycophenolate mofetil and the interleukin-2 (IL-2) receptor antagonist basiliximab. In the event of renal disease in the post-orthotopic liver transplant period, modifications to the immunosuppression regimen including CNI dose reduction with the addition of MMF, were performed.

No donor organs were obtained from executed prisoners or other institutionalized people.

This study was approved by the ethics committee of our institution (GAS-2368-17-20) and conforms to the provisions of the Declaration of Helsinki. Requirement of informed consent was waived due to its observational nature.

Data collection and primary outcomes

Medical records of all patients were examined to extract the following demographic and clinical variables [gender, age, cirrhosis etiology, presence of ascites and encephalopathy, vasopressor use, PaO_2/FiO_2 or SpO_2/FiO_2 relation, requirement of mechanical ventilation and precipitant event (bacterial infection, gastrointestinal hemorrhage, active alcoholism, other or unknown)]. Laboratory data measured at the time of LT necessary to determine disease severity and for the computation of the MELD-Na score was further registered: total bilirrubin (mg/dL), creatinine (mg/dL), INR, and leukocyte count ($\times 10^9$/L). Our primary outcomes of interest were: the development of immediate posttransplant infectious complications, defined as any type of nosocomial-acquired, donor-derived or surgery-related infection presented during the immediate hospital stay following LT until the patients' discharge; the development of any type of immediate postoperative complication according to Clavien-Dindo classification[15]; and post-LT survival at 1 year and 6 years. Similarities in donor liver graft quality were assessed by evaluation of the donor risk index (DRI[16]) which considers the donor's age, height, race and cause of death, donation after cardiac death, split/partial graft, organ allocation and cold ischemia time.

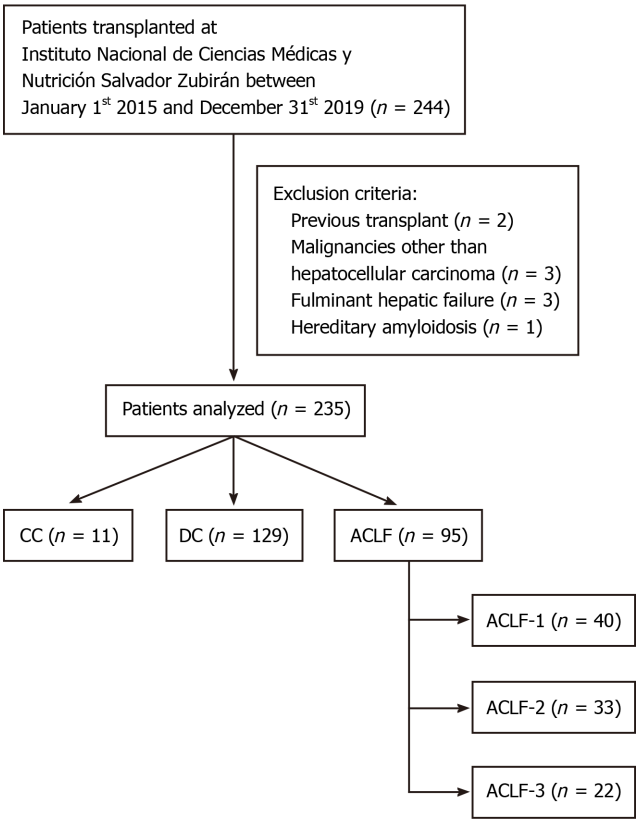

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Figure 1 Flowchart of the patients analyzed in this study. CC: Compensated cirrhosis; DC: Decompensated cirrhosis; ACLF: Acute-on-chronic liver failure.

Statistical analyses

Results of categorical variables are presented as frequencies and percentages, and as means and standard deviations or medians with interquartile range (IQR) for normally or not normally distributed continuous variables, respectively. Univariate statistical comparisons between categorical variables were performed with Pearson's Chi-squared test or Fisher's exact test and between continuous variables with the analysis of variance test or the Kruskal-Wallis test according to the normal distribution. Paired *t*-tests were carried out between disease severity scores among ACLF groups. Posttransplant survival was analyzed with the Kaplan-Meier method and survival curves were compared with the log-rank test. Statistical analyses were done with SPSS version 28.0 for Windows, IBM Corp., Armonk, NY, United States and survival curves were plotted using R version 4.1.0 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org) with the survminer package. Statistical significance was considered at a P value less than 0.05.

RESULTS

Non-ACLF and ACLF patient characteristics

A total of 235 patients that underwent LT from 2015 to 2019 were included in this study, of which 95 (38.9%) fulfilled ACLF criteria, 129 (52.9%) were classified as DC, and 11 (4.5%) as CC (Table 1). When compared to the CANONIC study, we identified an overall younger population with ACLF patients being even younger than those with DC and CC [50.0 years (IQR 37.0-59.0) *vs* 52.0 years (IQR 43.0-61.0) and 57.0 years (IQR 53.0-59.0), respectively; $P = 0.02$]. Autoimmune etiologies (autoimmune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis and overlapping syndromes) were the most frequent in ACLF patients (44.2% *vs* 27.9% DC, and 9.1% CC; $P < 0.01$), whereas the leading cause in DC and CC patients was HCV infection (72.7% CC, 32.6% DC, and 13.7% ACLF; $P < 0.0001$). With regard to comorbidities, no statistical differences were observed between cirrhosis groups for frequencies of either type 2 diabetes mellitus or primary hypertension ($P = 0.44$ and $P = 0.06$, respectively). ACLF patients had the highest MELD-Na score (25 ± 6 *vs* 19 ± 4 and 11 ± 3, DC and CC respectively), and accordingly the highest bilirubin and creatinine values. The presence of clinical ascites and encephalopathy (including West-Haven grade 3-4 encephalopathy) was also significantly higher in ACLF patients (Table 1).

Table 1 Patient characteristics (*n* = 235)

	CC, *n* = 11 (4.5%)	DC, *n*= 129 (52.9%)	ACLF, *n* = 95 (38.9%)	*P* value
Male gender, *n* (%)	6 (54.5)	64 (49.6)	47 (49.5)	0.95
Age, yr	57.0 (53.0-59.0)[a]	52.0 (43.0-61.0)[c]	50.0 (37.0-59.0)[a,c]	0.02
Liver cirrhosis etiology, *n* (%)				
Autoimmune	1 (9.1)[a]	36 (27.9)[c]	42 (44.2)[a,c]	< 0.01
HCV	8 (72.7)[a,b]	42 (32.6)[b,c]	13 (13.7)[a,c]	< 0.0001
Alcoholic liver disease	1 (9.1)	11 (8.5)	9 (9.5)	0.93
NASH	0 (0.0)	7 (5.4)	6 (6.3)	0.88
Cryptogenic	1 (9.1)	22 (17.1)	18 (18.9)	0.76
Other[1]	0 (0.0)	11 (8.5)	7 (7.4)	0.84
Comorbidities, *n* (%)				
Type 2 diabetes mellitus	3 (27.3)	22 (17.1)	13 (13.7)	0.44
Primary hypertension	2 (18.2)	20 (15.5)	6 (6.3)	0.06
Pre-transplant clinical data				
MELD-Na	11 ± 3[a,b]	19 ± 4[b,c]	25 ± 6[a,c]	< 0.0001
Total bilirubin (mg/dL)	1.18 (1.03-1.45)[a,b]	3.39 (2.3-5.46)[b,c]	7.70 (4.14-16.63)[a,c]	< 0.0001
INR	1.1 (1.1-1.2)[a,b]	1.5 (1.3-1.7)[b]	1.5 (1.3-2.0)[a]	< 0.0001
Serum creatinine (mg/dL)	0.67 (0.57-0.71)[a]	0.73 (0.61-0.88)[c]	0.97 (0.75-1.35)[a,c]	< 0.0001
Leukocyte count (× 10^9/L)	2.9 (2.4-3)[a]	4 (3.1-5)[c]	5.4 (3.8-6.8)[a,c]	< 0.0001
Disease manifestations[2], *n* (%)				
Clinical ascites	0 (0.0)[a,b]	88 (68.2)[b,c]	88 (92.6)[a,c]	< 0.0001
Encephalopathy	0 (0.0)[a,b]	69 (53.5)[b,c]	80 (84.2)[a,c]	< 0.0001
Grade 3-4 encephalopathy (West- Haven)	0 (0.0)[a,b]	7 (5.4)[b,c]	37 (38.9)[a,c]	< 0.0001

[1]Includes secondary biliary cirrhosis, drug-induced liver injury, and congenital liver diseases.
[2]Disease manifestations during the whole clinical course until liver transplantation.
Comparisons between groups are shown with superscript letters.
[a]Significant difference between CC and ACLF.
[b]Significant difference between CC and DC.
[c]Significant difference between DC and ACLF.
ACLF: Acute-on-chronic liver failure; CC: Compensated cirrhosis; DC: Decompensated cirrhosis; HCV: Hepatitis C virus; INR: International normalized ratio; MELD Na: Model for End-Stage Liver Disease-Sodium; NASH: Non-alcoholic steatohepatitis.

When assessing the last ACLF event of these patients before undergoing LT, the majority were classified as ACLF grade 1 [*n* = 40 (42.1%)] followed by ACLF-2 [*n* = 33 (34.7%)] and ACLF-3 [*n* = 22 (23.2%)] (Table 2). Overall median time to LT since their ACLF event was 31 d (IQR 11.0-88.0). However, those with ACLF-1 had a significantly longer time to LT compared to ACLF-2 and ACLF-3 [54.0 (IQR 21.3-122.8) *vs* 31.0 (IQR 7.0-59.5) and 22.0 (IQR 9.5-46.8), respectively; *P* = 0.03]. Demographic data and etiologies were similar within these three groups, with autoimmune etiologies being the most frequent among all ACLF grades (*P* = 0.30). The most common ACLF precipitant overall were bacterial infections and the absence of an identifiable factor (unknown). Other precipitants including pharmacological and procedure-related complications, were more frequent in ACLF-1 patients (*P* = 0.01). Kidney OF was the only one that did not differ significantly between ACLF groups [18 (45.0%) ACLF-1, 11 (33.3%) ACLF-2, and 14 (63.6%) ACLF-3; *P* = 0.09], whereas liver, brain, coagulation, circulation and lung failure were significantly higher in patients with ACLF-3 (Table 2).

Parameters reflecting disease severity including MELD-Na, CLIF-C OF and CLIF-C ACLF scores, total bilirubin, INR and leukocyte count were higher in ACLF-3 and lower in ACLF-1. We observed a generalized improvement of clinical parameters in ACLF patients at the time of LT, with a concomitant reduction of the disease severity scores evaluated. For instance, MELD-Na decreased significantly among all three ACLF grades (*P* < 0.01), and an improvement in the CLIF-C OF score was also observed. Interestingly, the CLIF-C ACLF score became similar within ACLF-1, 2 and 3 patients with no significant difference among them (*P* = 0.18), as well as INR and leukocyte count (*P* = 0.05 and *P* = 0.92,

Table 2 Acute-on-chronic liver failure patients characteristics (*n* = 95)

	ACLF-1, *n* = 40 (42.1%)	ACLF-2, *n* = 33 (34.7%)	ACLF-3, *n* = 22 (23.2%)	*P* value
Male gender, *n* (%)	21 (52.5)	17 (51.5)	11 (50.0)	0.98
Age, yr	55.0 (39.8-60.0)	44.0 (36.5-53.0)	49.0 (37.5-59.3)	0.14
Time to LT since ACLF event	54.0 (21.3-122.8)[a,b]	31.0 (7.0-59.5)[b]	22.0 (9.5-46.8)[a]	0.03
Liver cirrhosis etiology, *n* (%)				
Autoimmune	14 (35.0)	17 (51.5)	11 (50.0)	0.30
HCV	9 (22.5)	2 (6.1)	2 (9.1)	0.14
Alcoholic liver disease	2 (5.0)	4 (12.1)	3 (13.6)	0.44
NASH	3 (7.5)	2 (6.1)	1 (4.5)	0.99
Cryptogenic	10 (25.0)	5 (15.2)	3 (13.6)	0.50
Other[1]	2 (5.0)	3 (9.1)	2 (9.1)	0.69
ACLF precipitant, *n* (%)				
Bacterial infection	14 (35.0)	16 (48.5)	11 (50.0)	0.39
Gastrointestinal hemorrhage	1 (2.5)	3 (9.1)	1 (4.5)	0.44
Active alcoholism	0 (0.0)	0 (0.0)	0 (0.0)	0.99
Other	10 (25.0)[a]	2 (6.1)	0 (0.0)[a]	0.01
Unknown	15 (37.5)	12 (36.4)	10 (45.5)	0.77
Organ failures, *n* (%)				
Liver	14 (35.0)[a,b]	22 (66.7)[b]	16 (72.7)[a]	< 0.01
Kidney	18 (45.0)	11 (33.3)	14 (63.6)	0.09
Brain	5 (12.5)[a]	10 (30.3)	11 (50.0)[a]	< 0.01
Coagulation	3 (7.5)[a,b]	12 (36.4)[b]	11 (50.0)[a]	< 0.001
Circulation	0 (0.0)[a,b]	8 (24.2)[b,c]	14 (63.6)[a,c]	< 0.0001
Lung	1 (2.5)[a]	3 (9.1)	7 (31.8)[a]	< 0.01
ACLF event clinical data				
MELD-Na	27 ± 4[a,b]	29 ± 5[b,c]	35 ± 4[a,c]	< 0.0001
CLIF-C OF	9 ± 1[a,b]	10 ± 1[b,c]	12 ± 2[a,c]	< 0.0001
CLIF-C ACLF	39 ± 8[a]	43 ± 6[c]	52 ± 6[a,c]	< 0.0001
Total bilirubin (mg/dL)	6.31 (2.99-12.91)[a,b]	13.07 (6.39-22.31)[b]	23.08 (10.56-27.76)[a]	< 0.001
INR	1.5 (1.2-1.9)[a,b]	1.9 (1.4-2.5)[b]	2.2 (1.7-2.8)[a]	< 0.001
Serum creatinine (mg/dL)	1.86 (1.18-2.27)[b]	0.93 (0.71-1.99)[b,c]	2.25 (1.42-2.87)[c]	< 0.01
Leukocyte count (× 10^9/L)	6.55 (4.73-9.43)[a]	6.60 (4.55-8.25)[c]	8.75 (6.88-13.05)[a,c]	< 0.01
Pre-transplant clinical data				
MELD-Na	23 ± 4[a]	25 ± 5[c]	29 ± 8[a,c]	< 0.0001
CLIF-C OF	8 ± 2[a]	9 ± 2	10 ± 2[a]	0.01
CLIF-C ACLF	37 ± 9	37 ± 8	41 ± 12	0.18
Total bilirubin (mg/dL)	4.58 (2.94-8.60)[a]	9.75 (5.40-16.62)	19.59 (5.48-34.11)[a]	< 0.01
INR	1.4 (1.3-1.7)	1.6 (1.3-2.2)	1.9 (1.4-2.6)	0.05
Serum creatinine (mg/dL)	0.96 (0.75-1.29)[a]	0.88 (0.72-1.17)[c]	1.23 (0.94-1.95)[a,c]	< 0.01
Leukocyte count (× 10^9/L)	4.40 (3.23-6.70)	4.80 (2.90-6.30)	4.75 (2.48-10.45)	0.92

[1]Includes secondary biliary cirrhosis, drug-induced liver injury, and congenital liver diseases.

Comparisons between groups are shown with superscript letters.
[a]Significant difference between CC and ACLF.
[b]Significant difference between CC and DC.
[c]Significant difference between DC and ACLF.
ACLF: Acute-on-chronic liver failure; CLIF-C OF: Chronic Liver Failure Consortium: Organ Failure score; CLIF-C ACLF: Chronic Liver Failure Consortium: acute-on-chronic liver failure score; HCV: Hepatitis C virus; INR: International normalized ratio; MELD Na: Model for End-Stage Liver Disease-Sodium; NASH: Non-alcoholic steatohepatitis.

respectively) (Table 2).

ACLF patients have a more complicated posttransplant stay, but comparable short and long-term survival

Although severity of cirrhosis clearly differed between CC, DC and ACLF patients, posttransplant outcomes were mostly similar. While total days at the intensive care unit (ICU) were comparable and non-significant among these groups, patients with ACLF had a significantly longer hospital stay [8.0 d (IQR 6.0-13.0) *vs* 6.0 d (IQR 3.0-7.0) and 7.0 d (IQR 4.5-10.0), CC and DC, respectively; $P = 0.01$]. The frequency of patients who developed any type of complication (Clavien-Dindo I-V complications[15]) during their immediate hospital stay following LT was also similar, however those with ACLF more commonly presented an infectious complication ($P < 0.01$) (Table 3). When comparing days of hospital stay and posttransplant outcomes between ACLF-grades no significant differences were observed, thus the clinical course after LT of ACLF-3 patients was similar to that of those with ACLF-1 and 2 (Table 4).

Assessment of posttransplant mortality revealed that ACLF, DC and CC patients have a comparable survival at 1 and 6 years after LT [87 (91.6%), 114 (88.4%), 11 (100%) at 1 year, respectively; $P = 0.60$. 80 (84.2%), 112 (86.8%), and 10 (90.9%) at 6 years, respectively; $P = 0.90$]. Early transplant mortality at the critical periods of 30 d and 3 mo was also non-significant ($P = 0.38$ and $P = 0.30$, respectively).

All groups received the same quality grafts as there were no significant differences between groups in the DRI ($P = 0.13$) (Table 5). Survival as assessed by Kaplan-Meier analysis showed no significant differences among groups ($P = 0.79$; Figure 2A). These analyses were additionally performed in the ACLF population by subdividing them into their severity grades and no significant differences were observed at 30-d and 3-mo mortality ($P = 0.17$ and $P = 0.65$, respectively), 1-year and overall survival ($P = 0.40$ and $P = 0.15$, respectively). Likewise, no differences were observed in the DRI index ($P = 0.08$) (Table 5). This was reflected in a non-significant Kaplan-Meier analysis ($P = 0.17$; Figure 2B), which confirms similar posttransplant outcomes even among ACLF-3 patients.

DISCUSSION

Despite controversies, LT has been increasingly encouraged in patients with ACLF, including those with the highest severity grade. Hemodynamic derangements and systemic inflammation may restrain clinicians from considering an ACLF patient as a candidate for this procedure; however, the decision is so urgent that mortality on the waiting list may be even higher than that of status-1a patients[17]. In support of LT benefit for critically ill patients, this study demonstrates that according to our single-center experience, posttransplant outcomes in ACLF are favorable and in fact comparable with those of CC and DC patients. Moreover, even when comparing between ACLF grades a worse prognosis was not observed in those with ACLF-3.

In contrast to the CANONIC study[2], our patient population was in general younger, and interestingly ACLF patients were also the youngest even though no differences were found by ACLF grade. However, the main etiology in this group was of autoimmune nature. Although autoimmune diseases in cirrhosis follow a progressive and complicated clinical course, autoimmune ACLF patients in our center showed non-significant posttransplant survival differences in comparison with non-ACLF patients regardless of ACLF grade, which goes accordingly to the reported excellent survival observed in ACLF patients with autoimmune etiology[18]. A clear clinical difference between ACLF, CC and DC patients was evident by a significantly higher MELD-Na score and leukocyte count at the time of the ACLF event. These two parameters along with the CLIF-C and CLIF C-ACLF decrease at the time of LT, indicating improvement of the ACLF syndrome and hence a more favorable profile that allowed eventual transplantation. Indeed, Kim *et al*[19] has previously reported that both lower MELD scores and no ACLF progression are considered independent factors associated with a high survival rate after LT. Moreover, we also observed that the CLIF-C ACLF score at the time of LT was now similar between ACLF grades, which may further explain improvement and thus equally excellent posttransplant outcomes within these subgroups.

Compared to other studies[9,20-22], ACLF-3 patients in our center benefited from an even greater 1-year survival rate (90.9%) which remained higher even after our 6 year follow-up (77.3%). There are several risk factors associated with worse 1-year posttransplant mortality in ACLF-3 patients, such as

Table 3 Posttransplant outcomes (*n* = 235)

	CC, *n* = 11 (4.5%)	DC, *n* = 129 (52.9%)	ACLF, *n* = 95 (38.9%)	*P* value
ICU stay (d)	2.0 (1.0-4.0)	2.0 (1.5-4.0)	3.0 (2.0-5.0)	0.05
Hospital stay (d)	6.0 (3.0-7.0)[a]	7.0 (4.5-10.0)	8.0 (6.0-13.0)[a]	0.01
Any type of complication, *n* (%)	7 (63.6)	105 (81.4)	85 (89.5)	0.05
Infectious complications, *n* (%)	1 (9.1)[a]	38 (29.5)[b]	47 (49.5)[a,b]	< 0.01
Complications (Clavien-Dindo), *n* (%)				
I	4 (36.4)	17 (13.2)	19 (20.0)	0.08
II	2 (18.2)	51 (39.5)	32 (33.7)	0.31
III	1 (9.1)	14 (10.9)	17 (17.9)	0.27
IV	0 (0.0)	9 (7.0)	12 (12.6)	0.23
V	0 (0.0)	14 (10.9)	5 (5.3)	0.24

Comparisons between groups are shown with superscript letters.
[a]Significant difference between CC and ACLF.
[b]Significant difference between CC and DC.
[c]Significant difference between DC and ACLF.
ACLF: Acute-on-chronic liver failure; CC: Compensated cirrhosis; DC: Decompensated cirrhosis; ICU: Intensive care unit.

Table 4 Acute-on-chronic liver failure posttransplant outcomes (*n* = 95)

	ACLF-1, *n* = 40 (42.1%)	ACLF-2, *n* = 33 (34.7%)	ACLF-3, *n* = 22 (23.2%)	*P* value
ICU stay (d)	3.0 (2.0-4.0)	3.0 (2.0-6.0)	3.0 (2.0-6.0)	0.68
Hospital stay (d)	8.0 (5.0-11.8)	8.0 (6.0-15.5)	6.0 (5.8-14.3)	0.54
Any type of complication, *n* (%)	35 (87.5)	31 (93.9)	19 (86.4)	0.58
Infectious complications, *n* (%)	20 (50.0)	15 (45.5)	12 (54.5)	0.80
Complications (Clavien-Dindo), *n* (%)				
I	7 (17.5)	6 (18.2)	6 (27.3)	0.62
II	17 (42.5)	11 (33.3)	4 (18.2)	0.15
III	5 (12.5)	7 (21.2)	5 (22.7)	0.50
IV	4 (10.0)	6 (18.2)	2 (9.1)	0.49
V	2 (5.0)	1 (3.0)	2 (9.1)	0.61

ACLF: Acute-on-chronic liver failure; ICU: Intensive care unit.

older age (≥ 53 years), high pretransplant arterial lactate levels, mechanical ventilation and high leukocyte count (≤ 10 g/L)[23]. Contributing to the favorable outcome observed in our ACLF population, including those with ACLF-3, several of the above mentioned reported risk factors for worse posttransplant mortality were not present in our patients. First, a younger age characterized our ACLF population and clinical parameters were mostly stable across all severity grades at the time of LT. Leukocyte counts were higher than in DC and CC patients, but generally always lower than 10×10^9/L either during the ACLF event or at LT. While bacterial infections were the main ACLF precipitant followed by unknown factors, important differences regarding other cohorts can be found with the frequency of certain OFs. Respiratory failure which is a risk factor for lower posttransplant survival[11,20,23] was uncommon as lung OF seldom occurred. Instead, liver OF prevailed in those with severe ACLF although closely followed by extrahepatic OFs including kidney OF, which was the most frequent in those with ACLF-1.

Inevitably, ACLF patients will have a longer and more complicated hospital stay after LT as has been reported thus far[9,22]. This was true in our center, where the latter required more days of ICU and hospital stay. Posttransplant complications by the Clavien-Dindo classification[15], were not different between ACLF and non-ACLF patients (CC and DC) in accordance with a systematic review[22]. Despite this encouraging finding, infectious complications were specifically more common in the

Table 5 Posttransplant survival overall and by acute-on-chronic liver failure grade, *n* (%)

	CC, *n* = 11 (4.5%)	DC, *n* = 129 (52.9%)	ACLF, *n* = 95 (38.9%)	*P* value	ACLF-1, *n* = 40 (42.1%)	ACLF-2, *n* = 33 (34.7%)	ACLF-3, *n* = 22 (23.2%)	*P* value
DRI	1.41 (1.36-2.26)	1.38 (1.21-1.53)	1.32 (1.19-1.54)	0.13	1.43 (1.24-1.62)	1.27 (1.20-1.43)	1.38 (1.20-1.69)	0.08
30-d mortality	0 (0.0)	10 (7.8)	3 (3.2)	0.38	1 (2.5)	0 (0.0)	2 (9.1)	0.17
3-mo mortality	0 (0.0)	15 (11.6)	6 (6.3)	0.30	3 (7.5)	1 (3.0)	2 (9.1)	0.65
1-yr survival	11 (100)	114 (88.4)	87 (91.6)	0.60	35 (87.5)	32 (97.0)	20 (90.9)	0.40
Overall survival[1]	10 (90.9)	112 (86.8)	80 (84.2)	0.90	32 (80.0)	31 (93.9)	17 (77.3)	0.15

[1]Survival was analyzed until 6 years of follow-up, when the study was ended.
ACLF: Acute-on-chronic liver failure; CC: Compensated cirrhosis; DC: Decompensated cirrhosis.

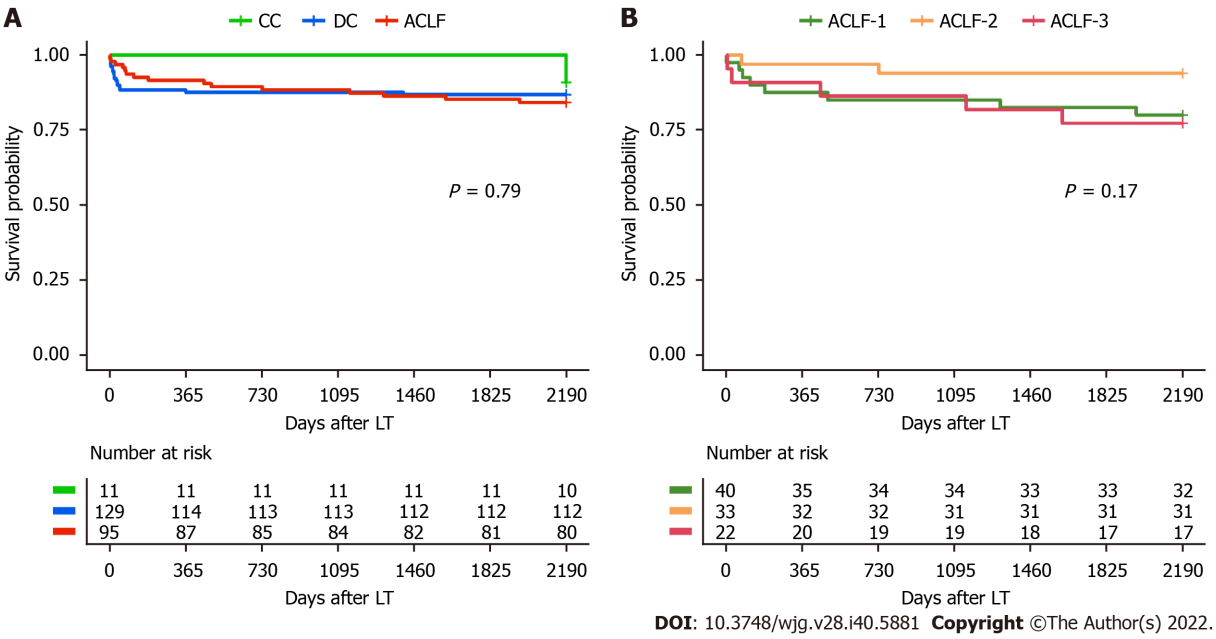

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Figure 2 Kaplan-Meier analyses for survival after liver transplant between compensated cirrhosis, decompensated cirrhosis, and acute-on-chronic liver failure (A), and between acute-on-chronic liver failure grades (B). LT: Liver transplant; CC: Compensated cirrhosis; DC: Decompensated cirrhosis; ACLF: Acute-on-chronic liver failure.

former, occurring in over half of them, which is also in agreement with the study of Artru *et al*[9]. This may warrant a more directed antibiotic regimen in ACLF patients and physicians should be aware of this frequent outcome to promote a longer posttransplant survival. Interestingly, infections were equally prevalent in ACLF-3 patients according to our experience, which may be due to the similar pretransplant profile identified among severity grades including non-significant CLIF-C ACLF score differences. A good donor liver graft quality which was comparable between CC, DC and ACLF patients is another factor that may have contributed to an overall excellent outcome; however, optimal graft quality must not impede the decision for LT given its lesser impact compared to early transplantation, as has been recently reported[24]. Overall, our results encourage further transplantation in those with ACLF, considering that this procedure is the only effective treatment option and that survival was not significantly different compared to patients with less advanced cirrhosis, despite a more complicated posttransplant clinical course.

This study is limited by its retrospective nature and its single-center design; hence, findings must be compared to those of other authors. We report here the experience of one of the largest transplant centers in Mexico; however, demographics in this center will certainly vary with those seen in the rest of the country. This may explain the high proportion of autoimmune patients compared to HCV or alcoholic hepatitis. Regardless, during the five-year study period we have found a comparable proportion of ACLF patients who undergo LT, whose disease severity is markedly different from CC and DC patients. In spite of these differences, we observed a clear LT benefit as has been supported by

previous studies.

CONCLUSION

In conclusion, out of 235 liver transplantation procedures that were carried out between 2015 and 2019 in our center, 38.9% corresponded to ACLF patients. Although important clinical differences were found with non-ACLF patients (CC and DC) and among each other when divided by severity grade, posttransplant survival was uniformly excellent. A longer hospital stay and frequency of infectious complications is to be expected, however, this should not restrain the decision to transplant those with ACLF. Furthermore, our observations support benefit even in the most critically ill patients (ACLF-3), given comparable 1-year and 6-year survival rates.

ARTICLE HIGHLIGHTS

Research background

Currently, liver transplantation (LT) is the only definitive therapeutic measure for patients with cirrhosis, albeit with the implied risks including posttransplant complications and the long-term use of immunosuppressive drugs. However, these patients benefit in general from excellent posttransplant survival. The benefit and survival of this procedure for patients with more advanced cirrhosis such as those with acute-on-chronic liver failure (ACLF), still remains controversial, with some reports showing a clear benefit, while others reporting lower short and long-term survival after transplant.

Research motivation

In order to contribute to the current literature regarding the benefit of LT even in those with more severe diseases, we evaluate the immediate posttransplant outcomes and compared the posttransplant survival in patients stratified by disease severity.

Research objectives

To assess immediate posttransplant outcomes and compare the short (1 year) and long-term (6 years) posttransplant survival among cirrhotic patients stratified by disease severity.

Research methods

We included cirrhotic patients undergoing liver transplantation between 2015 and 2019 and categorized them into compensated cirrhosis (CC), decompensated cirrhosis (DC), and ACLF. ACLF was further divided into severity grades. Medical records of all patients were examined to extract demographic and clinical variables as well as laboratory data measured at the time of LT and in the posttransplant period. Our primary outcomes of interest were: the development of immediate posttransplant infectious complications, defined as any type of nosocomial-acquired, donor-derived or surgery-related infection presented during the immediate hospital stay following LT until the patients' discharge; the development of any type of immediate postoperative complication according to Clavien-Dindo classification; and post-LT survival at 1 year and 6 years. Posttransplant survival was analyzed with the Kaplan-Meier method and survival curves were compared with the log-rank test.

Research results

A total of 235 patients underwent liver transplantation (CC = 11, DC = 129 and ACLF = 95). Patients with ACLF had a significantly longer hospital stay and developed more infection-related complications. Posttransplant survival at 1- and 6-years was similar among groups. When ACLF patients were stratified according to ACLF grade, similar intensive care unit and hospital stay lengths were found, as well as comparable frequencies of overall and infectious posttransplant complications. Despite that, there was no survival difference between ACLF grades at 1 year and 6 years.

Research conclusions

Patients may benefit from liver transplantation regardless of the cirrhosis stage. Despite having a longer hospital stay and a higher frequency of infectious complications, ACLF patients have excellent and comparable 1 and 6-year survival rates.

Research perspectives

A multicenter study would be required to determine the value of LT in advanced disease patients such as those with ACLF according to disease etiology.

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FOOTNOTES

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