Role of the Immune System in NAFLD Related Hepatocarcinogenesis

Tim F. Greten

Senior Investigator, Deputy Branch Chief TGMB
Co-Director NCI CCR Liver Cancer Program
Obesity: Public Health Crisis
Non-alcoholic fatty liver disease

- Most common type of liver disease
- Affects >65 Mio Americans
- Cost burden of US$103 billion annually within the US

- Insulin resistance, type II Diabetes, increased de-novo lipogenesis

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Simple steatosis

Gut microbiota

Adipose tissue inflammation

Oxidative stress

Hepatocyte Apoptosis

Hepatic inflammation

NASH

Fibrosis Cirrhosis

HCC
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Center for Cancer Research
NAFLD and HCC

modified from Cohen et al. (2011) Science; 332: 1519–23
MYC transgenic HCC mouse model

LAP-tTA/TRE-MYC

Doxycycline → HCC

MCD induced Non-Alcoholic Steatohepatitis (Choline Methionine deficient diet)

- Choline Methionine
  - Phosphatidylcholine synthesis
  - VLDL secretion

MCD diet

normal

NASH

1. Lipid accumulation.
2. Oxidation

VLDDL export
NASH promotes hepatocarcinogenesis

**MYC mice**

**MYC-CTR** vs **OFF-CTR**

- MYC ON
- 4, 6, 10, 14 wks

**Surface nodules/liver**

- MYC ON vs OFF-CTR
- Sacrifice at 10 and 14 wks
NASH promotes hepatocarcinogenesis

Ma et al. (2016) Nature 531:253
Selective CD4+ T cell loss in mice with NASH
Selective CD4+ T cell loss in mice with NASH
Selective CD4$^+$ T cell loss in mice with NASH
Selective CD4$^+$ T cell loss in the liver of mice with NASH

Liver

Spleen

Selective CD4$^+$ T cell loss in the liver of mice with NASH
DEN
2            6                                                                      32  wks
± HF/CDAA
sacrifice
Selective CD4+ T cell loss can be found different NAFLD models

![Graph showing CD4+ T cells per gram liver in different models.]

**DEN** (intrahepatic)

**Tumor free** (intrahepatic)
Differential infiltration of lymphocytes into obese adipose tissue (epididymal fat pads)

Intrahepatic CD4+ T cells die in NASH/myc mice

**MYC-CTR**

**MYC-MCD**

- Annexin V vs. 7AAD
- 7AAD: 84.9 vs. 4.98
- Annexin V: 0.317 vs. 22.2
- % Annexin V CD4+ T cells:
  - MYC-CTR: 10 ± 2
  - MYC-MCD: 30 ± 5

*Statistically significant difference.*
Hepatocytes from mice with NASH cause selective CD4\(^+\) T cell death
Linoleic acid (C18:2) cause selective CD4+ T cell loss
## Mouse diet composition

<table>
<thead>
<tr>
<th>Product</th>
<th>D10001 (AIN-76A)</th>
<th>D15052401 (2% LA)</th>
<th>D15052402 (12% LA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm%</td>
<td>Kcal%</td>
<td>gm%</td>
</tr>
<tr>
<td>Protein</td>
<td>20</td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>66</td>
<td>68</td>
<td>37</td>
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<tr>
<td>Fat</td>
<td>5</td>
<td>12</td>
<td>27</td>
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<tr>
<td>Kcal/gm</td>
<td>3.90</td>
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<tr>
<td>Corn Oil</td>
<td>50</td>
<td>450</td>
<td>0</td>
</tr>
<tr>
<td>Safflower Oil</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Coconut Oil</td>
<td>0</td>
<td>0</td>
<td>194</td>
</tr>
</tbody>
</table>
Linoleic acid enriched diet causes CD4\(^+\) T cell loss
Safflower Oil Benefits

- Reduces Cholesterol
- Reduces Blood Sugar Levels
- Enhances Hair Growth
- Nourishes Dry Skin

http://www.buzzle.com/articles/safflower-oil-benefits.html
Microarray analysis of linoleic acid-treated T cells

Splenocytes
- CD4 T
- CD8 T

Splenocytes + Linoleic acid
- CD4 T
- CD8 T

CD4 T cells LA vs. Control (Ingenuity analysis)

<table>
<thead>
<tr>
<th>Top Canonical Pathways</th>
<th>p-value</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative Phosphorylation</td>
<td>1.22E-10</td>
<td>18/83 (0.217)</td>
</tr>
<tr>
<td>Mitochondrial Dysfunction</td>
<td>2.33E-09</td>
<td>21/135 (0.156)</td>
</tr>
<tr>
<td>EIF2 Signaling</td>
<td>4.44E-05</td>
<td>16/155 (0.103)</td>
</tr>
<tr>
<td>TCA Cycle II (Eukaryotic)</td>
<td>3.67E-04</td>
<td>5/20 (0.25)</td>
</tr>
<tr>
<td>Regulation of eIF4 and p70S6K Signaling</td>
<td>1.08E-03</td>
<td>12/130 (0.092)</td>
</tr>
</tbody>
</table>
CPT1 dependent induction of CD4+ T cell death upon C18:2 incubation

Genes of electron transporter chain complexes

CPT1: Carnitine palmitoyltransferase I
Mitochondrial FFA metabolism

CPT1: rate-limiting enzyme to transfer LCFA into mitochondria

Long-chain fatty acids (LCFA): fatty acids with aliphatic tails 13 to 21 carbons

CPT1 has 3 isoforms:
CPT1A: liver, kidney, leucocytes, fibroblasts;
CPT1B: muscle, white adipose tissue;
CPT1C: Brain, testes.

Genes of electron transporter chain complexes
CPT1 dependent induction of CD4+ T cell death upon C18:2 incubation

Gene expression

Cell death

FFA oxidation
C18:2 increases mitochondrial ROS in CD4+ T cells
CD4$^+$ and CD8$^+$ T cells are equally susceptible to ROS mediated cell death.
N-acetyl cysteine treatment prevents CD4+ T cell loss

**in vitro**

<table>
<thead>
<tr>
<th></th>
<th>CD4</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-CD4</td>
<td></td>
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</tr>
</tbody>
</table>

**in vivo**

<table>
<thead>
<tr>
<th></th>
<th>CD4 T cells/g liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
</tr>
<tr>
<td>NAC</td>
<td></td>
</tr>
<tr>
<td>NAC + anti-CD4</td>
<td></td>
</tr>
</tbody>
</table>

* Data indicate significant differences.
N-acetyl cysteine treatment prevents CD4$^+$ T cell loss
CD4+ T cells, C18:2 and NASH in patients
Ma et al. (2016) Nature 531:253
Linolic acid probes colocalize with mitochondria

C18:2 upregulates CPT gene expression in murine T lymphocytes 

in vitro

CPT1a

CPT1b

CPT2

CD4 T cells

CD8 T cells
C18:2 upregulates CPT gene expression in murine T lymphocytes

*CPT1a*  
*CPT1b*  
*CPT2*

**Liver**  
**Spleen**

**Fold Induction**  

![Graph showing CPT gene expression in murine T lymphocytes](image)
Pharmacological PPAR-α blockade reverses C18:2 mediated CPT upregulation
Pharmacological PPAR-α blockade reverses C18:2 mediated CD4 T cell death

Mitochondrial ROS

Apoptosis

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CPT inhibitor perhexilene reduces HCC incidence in MYC-ON mice fed with MCD diet
Metabolic events control T cell immunity in liver cancer

**Hepatocyte**

- **C18:2**
- **CPT1**
- **FAO**
- **TCA**
- **C18:2**
- **CD36 (FATP)**
- **PPAR α**
- **Acetyl CoA**
- **Electron transport chain**
- **Decoupling**
- **ROS**
- **Survival**
Adaptive and innate chronic inflammation in the liver leading to NASH and HCC

**Chronic inflammation (adaptive) and cytokines**

- CD4+ or CD8+ T cells, ILCs, Th17 cells, IgA+ B cells, NKT and NK cells

**Hepatic metabolic reprogramming**
- Reduced hepatic expression of genes involved in lipolysis, lipogenesis and β-oxidation

**Metabolic stress**
- ER stress
- Oxidative stress
- Increased ROS levels

**Hepatocyte cell death**

**HSC activation**

**Fibrosis and/or cirrhosis**

**HCC development**

**Chronic inflammation (innate)**

Immune surveillance (+/-)
Metabolic Activation of Intrahepatic CD8+ T Cells and NKT-Cells Causes Nonalcoholic Steatohepatitis and Liver Cancer via Cross-Talk with Hepatocytes

Dyslipidemia + choline deficiency (CD-HFD) → NKT-cell → LIGHT → hepatocyte changes (steatotic, hyaluronic acid) → aberrant hepatocyte.

CD8+ T cell and NKT-cell interactions lead to hepatocyte changes including steatosis and expression of hyaluronic acid, contributing to the development of nonalcoholic steatohepatitis and liver cancer.
Obesity: Public Health Crisis

17% of children and adolescents

More than 1 in 3 adults
Mutant MHC class II epitopes drive therapeutic immune responses to cancer

Mutant MHC class II epitopes drive therapeutic immune responses to cancer

Fatty liver impairs the efficacy of cancer vaccines

B6(Cg)-Tyrc-2J/J

Background

B6(Cg)-Tyrc-2J/J

Reg. chow

Intrahepatic injection

Sacrifice

day

-7

0

1 4 8 15 20

RNA-vaccination

MCD diet

Intrahepatic injection

Sacrifice

day

-7

0

1 4 8 15 20

RNA-vaccination
Fatty liver impairs the efficacy of cancer vaccines

Radiance (p/sec/cm²/sr)

- Control, reg. diet
- M30-vaccine, reg. diet

Control, reg. diet
M30-vaccine, reg. diet

0
2×10⁹
4×10⁹
6×10⁹
8×10⁹
D4 D8 D15 D20

ns

M30-vaccine, reg. diet
Fatty liver impairs the efficacy of cancer vaccines
Fatty liver impairs the efficacy of cancer vaccines

Hepatic CD4 T cells

Frequency of live lymphocytes (%)
M30 vaccine is effective in NAC treated mice on MCD diet

- **Control**
- **M30 vaccine**
- **Control /NAC**
- **M30 vaccine NAC**

**Radiance (p/sec/cm²/sr)**

- Days post injection: D4, D8, D15, D20

**Tumor : liver weight ratio**

- H20 C
- NAC C
- NAC aCD4

**CD4 T cell depletion**

- **ns**
- ***

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Anti-Ox40 treatment

Engagement of the OX-40 receptor in vivo enhances antitumor immunity

Currently 48 clinical trials registered targeting Ox-40

Weinberg et al. (2000) JI 164:2160-9
Fatty liver impairs the efficacy of anti-Ox40 treatment

Regular vs MCD diet

Tumor to liver weight-ratio

Reg. chow

MCD diet
Fatty liver impairs the efficacy of anti-Ox40 treatment

Regular vs MCD diet

MCD diet ± NAC

Tumor to liver weight-ratio

0.0
0.2
0.4
0.6
0.8

IgG
αOx40
IgG
αOx40

Reg. chow

MCD diet

MCD diet

IgG

αOx40

IgG

αOx40

NAC

NAC
Summary

- CD4 T cells are depleted in mice with fatty liver disease through a ROS mediated mechanism.

- This impacts the effect of cancer vaccines and anti-Ox40 treatment.

- NAC treatment reverses diet induced CD4 T cell loss and enhances treatment efficacy (M30 and anti-Ox40) in mice with fatty liver disease.
The microbiome and hepatocarcinogenesis

The figure illustrates the interaction between the microbiome and hepatocarcinogenesis. Key components include:

- **NASH**, **HBV**, and **HCV** leading to **Dysbiosis**, which further impacts **Leaky gut**.
- **Metabolites** and **MAMPs** contribute to the dysbiotic state.
- **TMAO** and **EtOH** can affect hepatocytes.
- **Secondary BA** (DCA) and **Primary BA** influence cellular processes.
- **Hepatocyte** proliferation is regulated by **LSEC**, **CXCL16**, and **NKT**.
- **HSC** and **Macrophage** activities are modulated by **SASP**, **Ereg**, and **TMAO**.
- Inflammation markers include **IL-1, IL-6, TNF**.

**SCFA+BA** and **Fiber + resistant starch** are highlighted as potential therapeutic targets.

*Schwabe & Greten J. Hepatology in press*
Gut microbiota control growth of liver tumors

MYC mice

H2O

14 weeks

Surface Nodules # / liver

0
10
20
30
40

H2O
Gut microbiota control growth of liver tumors

MYC mice

H2O

ABX

ABX/H2O

MYC-ON

14 weeks

Surface Nodules # / liver

H2O

ABX

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The gut microbiome controls growth of liver tumors

Intrasplenic B16

B6 mice

H2O  ABX

Intrasplenic B16

-3  0  1.5 weeks

Surface Mets # / Liver

H2O  ABX

A20 intravenous

Balb/c mice

H2O  ABX

i.v. A20

-3  0  3 weeks

Surface Mets # / Liver

H2O  ABX

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Lung tumors are not affected by Abx treatment
Hepatic NKT cells accumulate in Abx treated mice

![Graph showing cell counts of different cell types in EL4-H2O and EL4-ABX treated mice.](image-url)
NKT cells

- Share properties of both T cells and natural killer cells
- Recognize the non-polymorphic CD1d molecule
- CD1d binds self and foreign lipids and glycolipids.
- ~50% of murine intrahepatic lymphocytes
- Type I NKT cells express conserved Vα gene (Vα24 in humans and Vα14 in mice)
- CXCR6⁺
CXCR6+ NKT cells accumulate upon Abx treatment
Liver sinusoidal endothelial cells upregulate CXCL16 and mediate hepatic NKT accumulation

ABX

Gut microbiome

Liver CXCL16 (LSEC)

 Liver NKT

Liver tumor
Enterohepatic Bile Acid Circulation

1. Secreted bile salts consist of 95% old, recycled bile salts and 5% newly synthesized bile salts.
2. 95% of bile salts are reabsorbed by the small intestine.
3. Reabsorbed bile salts are recycled by enterohepatic circulation.
4. 5% of bile salts are lost in feces.
Hepatic Bile Acid Profile

Primary bile acid

Secondary/tertiary bile acid

nM/mg of liver tissue

H2O
ABX

Primary bile acid
Secondary/tertiary bile acid
Bile acids control CXCL16 expression in the liver
Bile acids control CXCL16 expression in the liver
Bile acids control CXCL16 expression in the liver
Bile acids control liver tumor growth

BALB/c mice

-3
0
3 weeks

Vancomycin

A20 i.v.

-MCA gavage

H2O

Vanco

Vanco+ H-MCA

Kill
Bile acids control liver tumor growth
Commensal bacteria mediate bile acid mediated NKT cell accumulation
Clostridium species mediate bile acid mediated NKT cell accumulation
C. scindens treatment ameliorates anti-tumor immunity in the liver in vancomycin treated mice
C. scindens treatment ameliorates anti-tumor immunity in the liver in vancomycin treated mice
Secondary BA
Primary BA
Microbiota
Fecal BA pool
Gut epithelium
Portal vein blood with prim. & sec. BA
NKT cell
CXCR6
LSEC
CXCL16
Portal vein
Tumor
Portal vein
Blood
with
prim.
&
sec.
BA

Ma et al. (2018) Science 360:eaan5931
Phase II Study of Nivolumab (anti-PD1), Tadalafil and Oral Vancomycin in Patients with Refractory Primary Hepatocellular Carcinoma or Liver Dominant Metastatic Cancer from Colorectal or Pancreatic Cancers
NCI CCR Liver Cancer Program: Special Conference on Tumor Metabolism
October 28-29, 2019; NIH Bethesda Campus, Maryland, U.S.A.

Conference Website:
https://ncifrederick.cancer.gov/events/conferences/2019NCICCRLCP

• Updates on research progress and directions
• Focus on tumor metabolism
• Experts and outstanding speakers in the field
• Foster collaboration and team science

Registration is OPEN and FREE
Abstract submission for posters is OPEN; Due by June 25, 2019
Best judged abstracts will be selected for oral presentations and travel awards!

Organizers: Tim Greten & Xin Wang (NCI CCR LCP Co-Directors)
Bin Gao, Mitchell Ho and Katherine McGlynn
Greten Lab

Chi Ma
Firouzeh Korangy
Bernd Heinrich
Simon Wabitsch (DFG)
Benjamin Ruf
Walter Lai (NIDDK)
Umberto Rosato
Laurence Diggs (Surg)
Qianfei Zhang
Varun Subramanyam
Linda Cao
Sophie Wang

Former lab members
Su Jong Yu
Qiong Fu
Zachary Brown
Miaojun Han

Clinical Team

Changxing Xie
Cecilia Monge Bronez
Donna Mabry-Hones
Suzanne Fioravanti
Melissa Walker

Former members
Austin Duffy
Gagandeep Brar
Charalampos Floudas

Laboratory of Pathology
David Kleiner

Interventional Radiology
Brad Wood

NIH Collaborators

Cancer and Inflammation Program
Giorgio Trinchieri,
Soumen Roy, Wuxing Yuan,
Vishal Thovarai,
Shurjo K. Sen

Vaccine Branch
Jay Berzofsky, Masaki Terabe

Laboratory of Human Carcinogenesis
Xin Wang, Juling Ji,
Snorri Thorgeirsson
Valerie Fako, Lichun Ma

Laboratory of Molecular Biology
Mitchell Ho, Dan Li, Nan Li

National/International Collaborators

North Carolina State University
Casey Theriot

Stanford University
Dean Felsher

TIGER Consortium
Mathuros Ruchirawat

University of Aachen, Germany
Thomas Ritz

DKFZ, Heidelberg, Germany
Christian Rupp
Thomas Longerich