AUS DEM LEHRSTUHL
FÜR INNERE MEDIZIN I
PROF. DR. MARTINA MÜLLER-SCHILLING
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

DEFICIENCY OF INTESTINAL MUCIN-2 AMELIORATES EXPERIMENTAL ALCOHOLIC LIVER DISEASE IN MICE

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

der
Fakultät für Medizin
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Das Fehlen von intestinalem Muzin-2 verbessert experimentelle alkoholische Lebererkrankung in Mäusen

In dieser deutschen Zusammenfassung des Orginalartikels wurden einzelne Passagen sowie Abbildungen ausgelassen, die im Originalartikel auf englisch weiter unten einsehbar sind. Die Abbildungen I A-B; II A-C; III A-C; III D-F; IV; V A-C; VI sind dem Originalartikel entnommen und entsprechen jeweils den Figures 1 A-B; 2 A-C; 5 A und D-E; 5 F links, Mitte und rechts; 6 C; 7 A-C; 8. Einzelne Methoden sind im Text erwähnt, der der restliche Methodenteil ist ausführlicher im Originalartikel erklärt. Die in der deutschen Zusammenfassung angegebenen Referenznummern entsprechen denen des Originalartikels.

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Überblick

Die intestinale Schleimschicht schützt das Epithel vor schädlichen Stoffen, Viren und pathogenen Bakterien, die sich im Magen-Darm-Trakt befinden. Sie besteht aus Muzinen, insbesondere Muzin-2 (Muc2), welche von Becherzellen des Darmes sezerniert werden. Experimentelle alkoholische Lebererkrankung erfordert eine Translokation von Bakterienbestandteilen über die Darmwand in die systemische Blutzirkulation, welche entzündliche Prozesse in der Leber bewirken und zur Steatohepatitis beitragen. Wir untersuchten die Rolle der Schleimschicht des Darmes, im Speziellen die von Muzin-2, in der Entstehung der experimentellen alkoholischen Lebererkrankung in Mäusen. Diese Erkrankung induzierten wir in Wildtyp-Mäusen und Mäusen, denen Muzin-2 fehlte ($Muc2^{-/-}$), mit Hilfe des Tsukamoto-French-Modells, in dem den Mäusen kontinuierlich entweder isokalorische Nahrung oder Alkohol intragastrisch appliziert wurde. $Muc2^{-/-}$ Mäusen zeigten einen geringeren Grad an durch Alkohol verursachte Leberschädigung und Fettleber, als dies Wildtyp-Mäuse entwickelten. Vor allem offenbarten $Muc2^{-/-}$ Mäuse signifikant niedrigere Plasmalevel von Lipopolysacchariden als Wildtyp-Mäuse nach Alkoholadministration. Im Gegensatz zu Wildtyp-Mäusen waren $Muc2^{-/-}$ Mäuse vor Alkohol-assoziierten Veränderungen der Darmflora geschützt. Die antimikrobiellen Proteine regenerating islet-derived 3 beta und gamma (Reg3b und g) waren im Vergleich zu Wildtyp-Mäusen auf signifikant höheren Niveaus im Jejunum von $Muc2^{-/-}$ Mäusen exprimiert, denen jeweils isokalorische Nahrung bzw. Alkohol verabreicht wurde. Infolgedessen zeigten $Muc2^{-/-}$ Mäuse erhöhtes Abtöten kommensaler Bakterien und beugten einer intestinalen bakteriellen Überwucherung vor. Zusammenfassend sind $Muc2^{-/-}$ Mäuse geschützt vor einer intestinalen bakteriellen Überwucherung bzw. einer Fehlbesiedlung nach Alkoholadministration. Folglich treten weniger Bakterienbestandteile wie Endotoxin in den systemischen Blutkreislauf über und verursachen mithin einen geringeren Leberschaden.

Einleitung

Leberzirrhose ist die zwölfthäufigste Todesursache in den USA, und 48% von allen zirrhotischen Todesfällen stehen im Zusammenhang mit Alkohol (1). Alkoholische Lebererkrankung, oder Alcoholic Liver Disease (ALD), umfasst Steatosis hepatis, welche zur alkoholischen Hepatitis, Fibrose und Zirrhose fortschreiten kann (2). Es gibt starke Beweise für die Darm-Leber-Achse, die in ursächlicher Verbindung steht zu Alkohol-bedingter Lebererkrankung, in experimentellen Tiermodellen wie auch im Menschen. Die Permeabilität im Darm-Trakt ist größer in Menschen mit Alkoholkrankheit im Vergleich zu gesunden Menschen (3, 4). Einige Tierstudien zeigten, dass Ethanol die intestinale epitheliale Schutzfunktion durch den direkten Effekt von Alkohol und/oder seines Metaboliten Acetaldehyd zerstört (5). Die durch Ethanol begründete erhöhte Durchlässigkeit („leaky gut“) führt zu erhöhten Plasmalspiegeln von Lipopolysacchariden (LPS, auch als Endotoxin bezeichnet), einem Hauptbestandteil der äußeren Zellwand von Gram-negativen Bakterien, und mithin größerem Leberschaden (6-8). Endotoxämie ist statistisch häufiger in Patienten mit alkoholischer Lebererkrankung im Vergleich zu gesunden Menschen; die Plasma-LPS-Level korrelieren mit dem Ausmaß des Leberschadens in Patienten mit alkoholischer Hepatitis (9-12). Der überzeugendste Beweis für die Rolle von aus dem Darm kommendem Endotoxin stammt von Mäusen, die eine Gendeletion in der LPS-Signalkaskade besitzen. Toll-like receptor 4 (TLR4) Knock-out-Mäuse, das heißt Mäuse, denen dieser zelluläre LPS-Rezeptor fehlt, bzw. Mäuse ohne CD14, dem zellulären Ko-Rezeptor für LPS, sowie Mäuse, denen intrazelluläre Moleküle in der Signaltransduktionskaskade nach dem LPS-Rezeptor fehlen, sind resistent gegen Alkohol-induzierten Leberschaden (13-15). Darüberhinaus reduziert die selektive Dekontamination des Verdauungstraktes mit nicht-absorbierbaren Antibiotika die Plasma-Endotoxinspiegel und verhindert experimentelle alkoholische Lebererkrankung (16-18). Obwohl eine Behandlung mit Antibiotika die Leberfunktion in Patienten mit Alkohol-bedingter Zirrhose verbessert, ist dies noch nicht eine etablierte Therapie (19).

Die intestinale Schleimschicht formt eine physische Barriere zwischen dem unterliegenden Epithel und dem Lumen des Verdauungstraktes, und schützt das Epithel vor Noxen, Viren und pathogenen Bakterien. Es besteht aus zwei Unterschichten: die innere Schicht liegt der Epithelschicht an und birgt keine Bakterien; die äußere Schicht ist leicht abwaschbar und ist kolonisiert mit Bakterien (20, 21). Die intestinale Schleimschicht ist zusammengesetzt aus Muzinen, die von intestinalen Becherzellen synthetisiert und sezerniert werden (22). Es existerieren zwei unterschiedliche Typen von Muzinen: sezernierte oder Gel-formende Muzine,

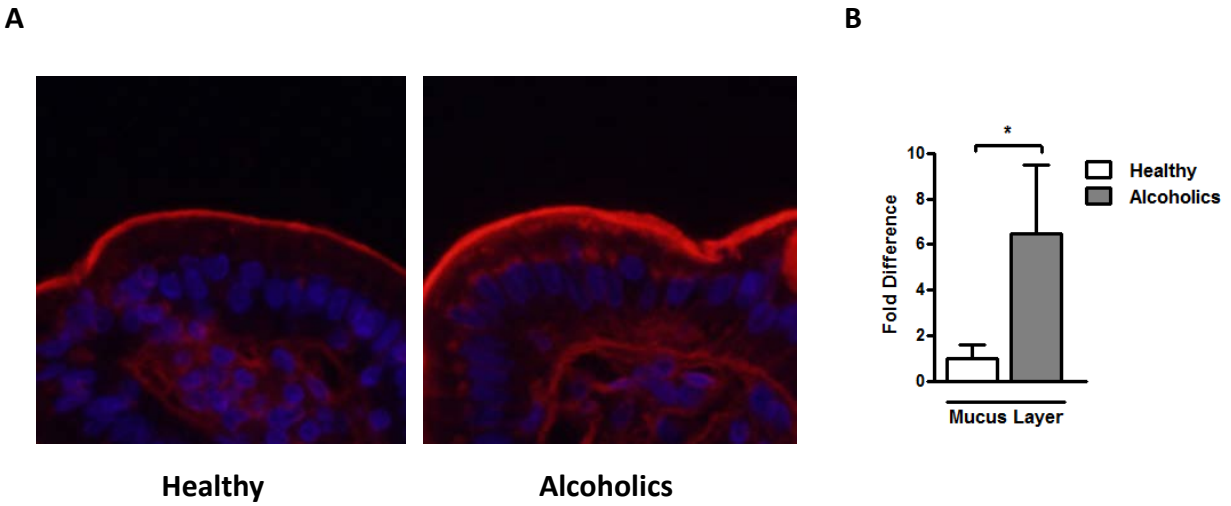
und Membran-gebundene Muzine. Es gibt drei gastrointestinale sezernierte Muzine (Muc2, Muc5AC und Muc6), welche typischerweise große, stark O-glykosylierte Glykoproteine sind, die zusammengesetzt zu Oligomeren zu den viskösen Eigenschaften der Schleimschicht des Verdauungstraktes beitragen (23). Die intestinalen Membran-gebundenen Muzine (Muc1, Muc3-4, Muc12-13 und Muc17) schützen gegen Pathogene, die die innere Schleimschicht penetrieren (24). Das sezernierte Hauptmuzin im Dünn- und Dickdarmbereich ist Muzin-2 (25). $Muc2^{-/-}$ Mäuse, das heißt Mäuse, denen Muzin-2 fehlt, besitzen ein höheres Risiko, ein kolorektales Karzinom zu entwickeln, und scheinen eine gestörte epitheliale Homeostase zu haben (25). Spezfische klinische Symptome wie spontane Kolitis hängen von dem jeweiligen genetischen Mausstamm ab (26).

Es wurde berichtet, dass sich die intestinale Mukusproduktion in Ratten nach Alkoholadministration erhöht (27). Jedoch, gibt es bis jetzt keine Patientendaten oder experimentelle Studien, die den funktionellen Beitrag der intestinalen Schleimschicht in alkoholischer Lebererkrankung untersuchte. Wir haben daher einen unvoreingenommenen Ansatz unternommen, die Rolle der intestinalen Schleimschicht zu untersuchen, und im Besonderen die von Muzin-2, indem wir ein Mausmodell der alkoholischen Leberläsion und Steatose nutzten.

Ergebnisse

Alkoholmissbrauch erhöht die Dicke der intestinalen Schleimschicht im Menschen. Es wurde berichtet, dass chronische Alkoholadministration die Gesamtmenge an Mukus im Dünndarm in Ratten erhöht (27). Wir haben dies im Menschen bestätigt. An Alkoholkrankheit leidende Menschen zeigen einen signifikanten Anstieg der Dicke der Schleimschicht in Biopsien im Duodenum im Vergleich zu gesunden Menschen. (Abb. I A-B).

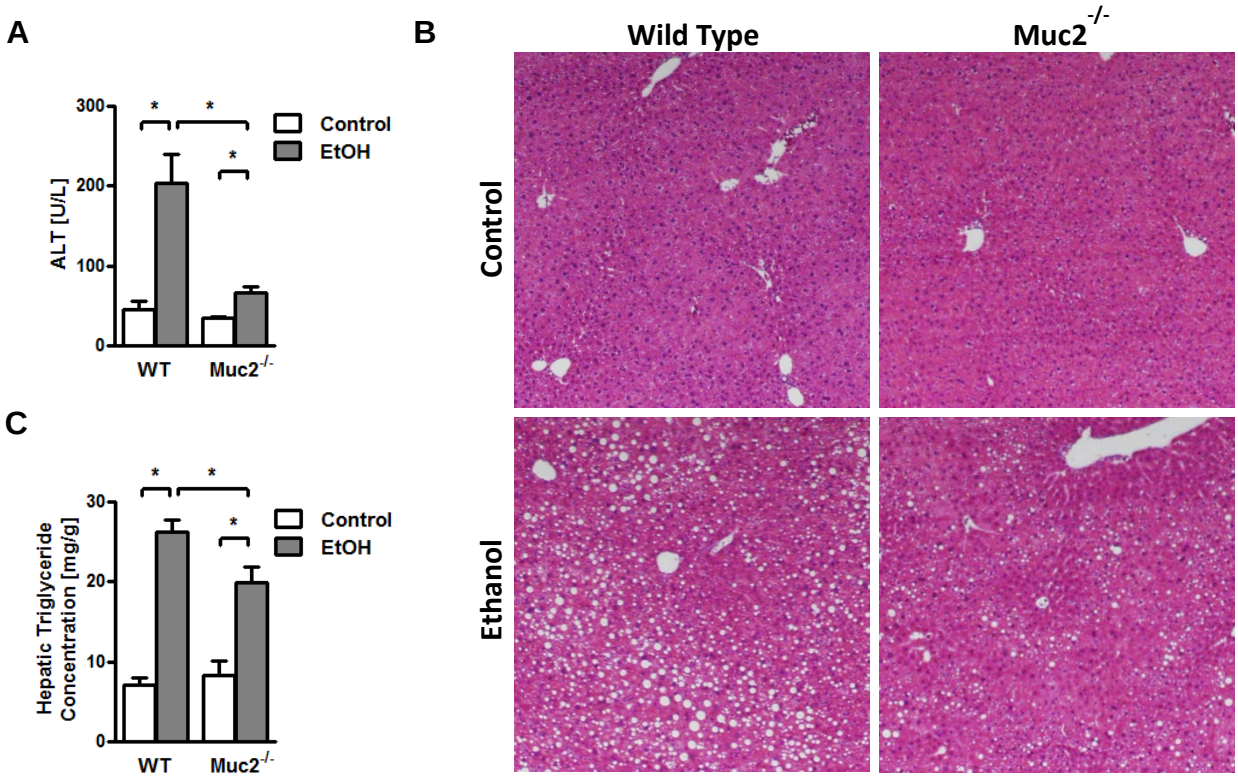
Abbildung I – Ethanol führt zu einer dickeren Darmschleimschicht



Muzin-2-defiziente Mäuse zeigen weniger Alkohol-induzierte Steatohepatitis. Um die Rolle der intestinalen Schleimschicht in experimenteller alkoholischer Lebererkrankung zu untersuchen, haben wir Mäuse mit genetischer Deletion des Muzin-2-Genes ($Muc2^{-/-}$) für die Experimente genutzt (25). Muzin-2 ist das Hauptmuzin, welches im Verdauungstrakt sezerniert wird (25) und sein Fehlen führt zu einer signifikant dünneren Schleimschicht; bei Wildtyp-Mäusen haben wir es nicht in der Leber oder in vom Knochenmark abstammenden Zellen nachweisen können, seine Expression ist hier weitaus am stärksten in Dick- und Dünndarm (s. Originalartikel weiter unten für Abbildungen). Somit haben wir ein kontinuierliches, intragastrisches Zuführungsmodell von Ethanol („Tsukamoto-French Mouse Model“) über eine Woche durchgeführt. Kontrollen waren Mäuse, denen isokalorische Nahrung intragastrisch verabreicht wurde. Die Alanin-Aminotransferase (ALT)-Level im Plasma als Maß der Leberschädigung waren signifikant niedriger in Alkohol-ernährten $Muc2^{-/-}$ Mäusen verglichen mit Wildtyp-Mäusen (Abb. II A). Ebenso waren nach Ethanoladministration die hepatische

Fettakkumulation signifikant geringer in Muc2$^{-/-}$ Mäusen, in Form von geringerer mikro- und makrovesikulärer Steatose (Abb. II B) sowie als Laborwert niedriger gemessene Triglyzeride in der Leber (Abb. II C).

Abbildung II – Das Fehlen von Muzin-2 verbessert experimentelle alkoholische Lebererkrankung



Der Metabolismus von Alkohol und die Expression von intestinalen Muzinen nach Ethanoladministration zeigt keinen signifikanten Unterschied zwischen Muzin-2-defizienten Mäusen und Wildtyp-Mäusen. Um diesen unterschiedlichen hepatischen Phänotyp zu erklären, untersuchten wir, ob das Fehlen von Muzin-2 die intestinale Absorption oder den hepatischen Metabolismus von Alkohol beeinflusst. Die Plasma-Alkoholspiegel, Alkohol dehydrogenase (Adh) und Cytochrom p450 Enzym 2E1 (Cyp2E1) – als Hauptenzyme in der Leber, um Alkohol zu metabolisieren und ihn zu Acetaldehyd umzuwandeln (29) – sowie Acetaldehyd-Level im Plasma konnten nicht das unterschiedliche Erscheinungsbild der Leber zwischen Muzin-2-defizienten Mäusen und Wildtyp-Mäusen nach Alkoholgabe erklären (Genaures s. Originalartikel). Desweiteren fragten wir, ob ein Fehlen von Muzin-2 eine

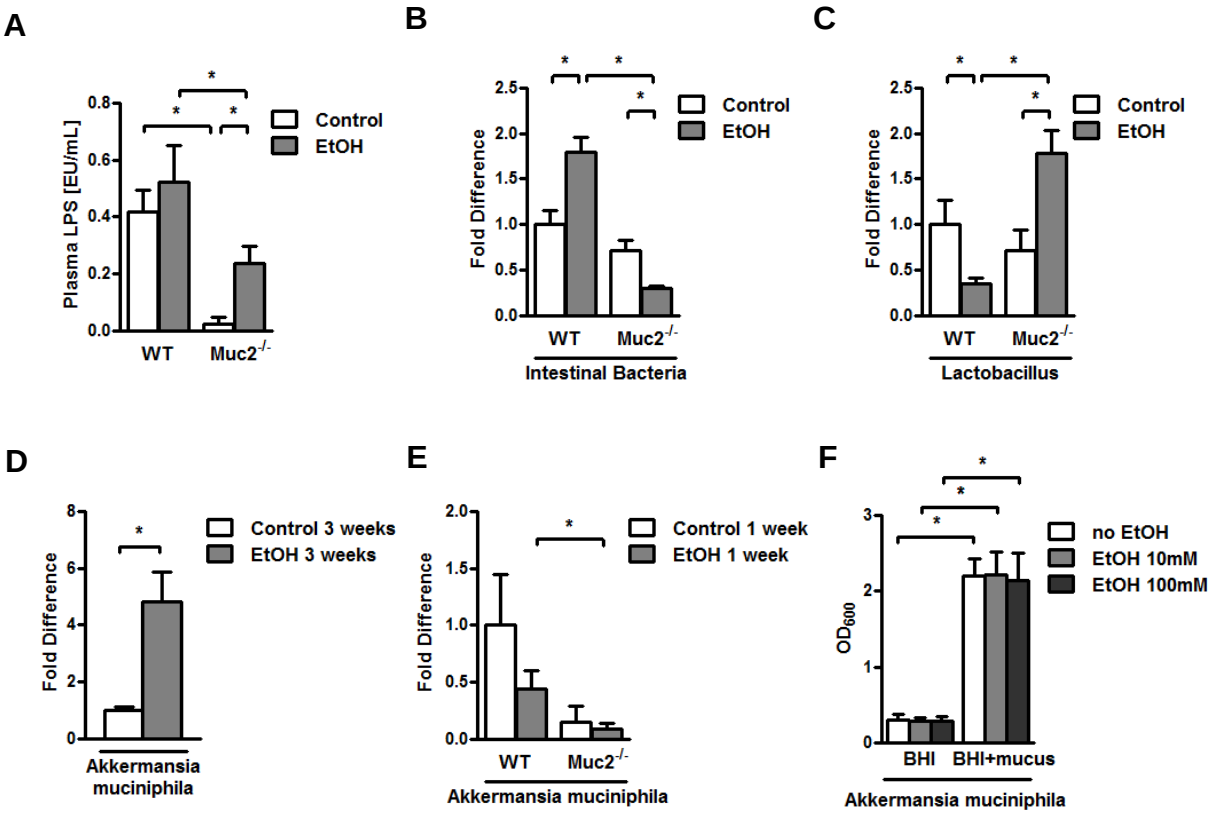
kompensatorische Hochregulation von anderen intestinalen Muzinen bewirken kann. Jedoch führte eine Ethanoladministration in Muc2$^{-/-}$ Mäusen nicht zu einer Veränderung der Dicke der intestinalen Schleimschicht oder der Expression von sezernierten Muzinen (wie Muzin-6) oder von Membran-gebundenen Muzinen (wie Muzin-1 und -4). (Genaueres s. Originalartikel).

Muzin-2-defiziente Mäuse zeigen niedrigere LPS-Plasmaspiegel und sind gegen Veränderungen der Darmflora nach Alkoholgabe geschützt. Alkoholische Steatohepatitis hängt von Endotoxin ab, das von intestinalen Bakterien stammt (2, 30). Da Muzin-2 im Darm jedoch nicht in der Leber exprimiert wird, untersuchten wir, ob die Translokation von bakteriellen Produkten aus dem Darm in die systemische Zirkulation von dem Fehlen von Muzin-2 beeinflusst ist. Nach einwöchiger Administration von Ethanol bzw. isokalorischer Nahrung offenbarten Muc2$^{-/-}$ Mäuse signifikant geringere systemische Endotoxin-Level als ihr jeweiliges Wildtyp-Pendant (Abb. III A). Veränderte intestinale Permeabilität oder ein quantitativer Abfall der intestinalen Mikroflora könnte dazu führen, dass weniger Endotoxin von dem Darm in die systemische Zirkulation gelangt. Wir haben deshalb die intestinale Permeabilität nach zweiwöchiger Alkohol- bzw. isokalorischer Nahrung (Lieber-DeCarli Modell) untersucht (31). Experimente mit Messung des fäkalen Albumins wie auch des oral verabreichten mit Fluorescein-Isothiocyanat (FITC) markierten Dextrans im Plasma zeigten beide eine signfikant erhöhte intestinale Permeabiltät in Muzin-2-defizienten Mäusen nach isokalorischer sowie alkoholischer Nahrung (Genaueres s. Originalartikel). Folglich zeigten Muc2$^{-/-}$ Mäuse trotz höhergradiger intestinaler Permeabilität eine geringere Translokation von bakteriellen Produkten.

Die Gesamtzahl der intestinalen Bakterien wurde mittels qPCR mit universalen 16S-rRNA-Bakterien-Primer-Sets quantifiziert. Wie schon von uns berichtet (28), führt intragastrische Ethanol-Administration zu intestinaler bakterieller Überwucherung in Wildtyp-Mäusen in Relation zu isokalorisch-ernährten Wildtyp-Mäusen. Interessanterweise sind Muc2$^{-/-}$ Mäuse vor intestinaler bakterieller Überwucherung geschützt (Abb. III B). Weitere Ergebnisse aus der Forschungsgruppe um Dr. Schnabl bezüglich der Alkohol-assoziierten Veränderungen des enterischen Mikrobioms (28) wurden bestätigt, so zum Beispiel eine signifikante Reduktion von *Lactobacillus* in Wildtyp-Mäusen nach einwöchiger intragastrischer Ethanolgabe im Vergleich zu Kontroll-Wildtyp-Mäusen. Muc2$^{-/-}$ Mäuse demonstrierten nicht nur einen Schutz vor einer Alkohol-induzierten Suppression von *Lactobacillus*, sie offenbarten sogar einen signifikanten Anstieg im Vergleich zu isokalorisch-ernährten Muc2$^{-/-}$ Mäusen (Abb. III C). Darüberhinaus haben wir wiederum Daten von Dr. Schnabl's Labor bestätigt, dass eine dreiwöchige

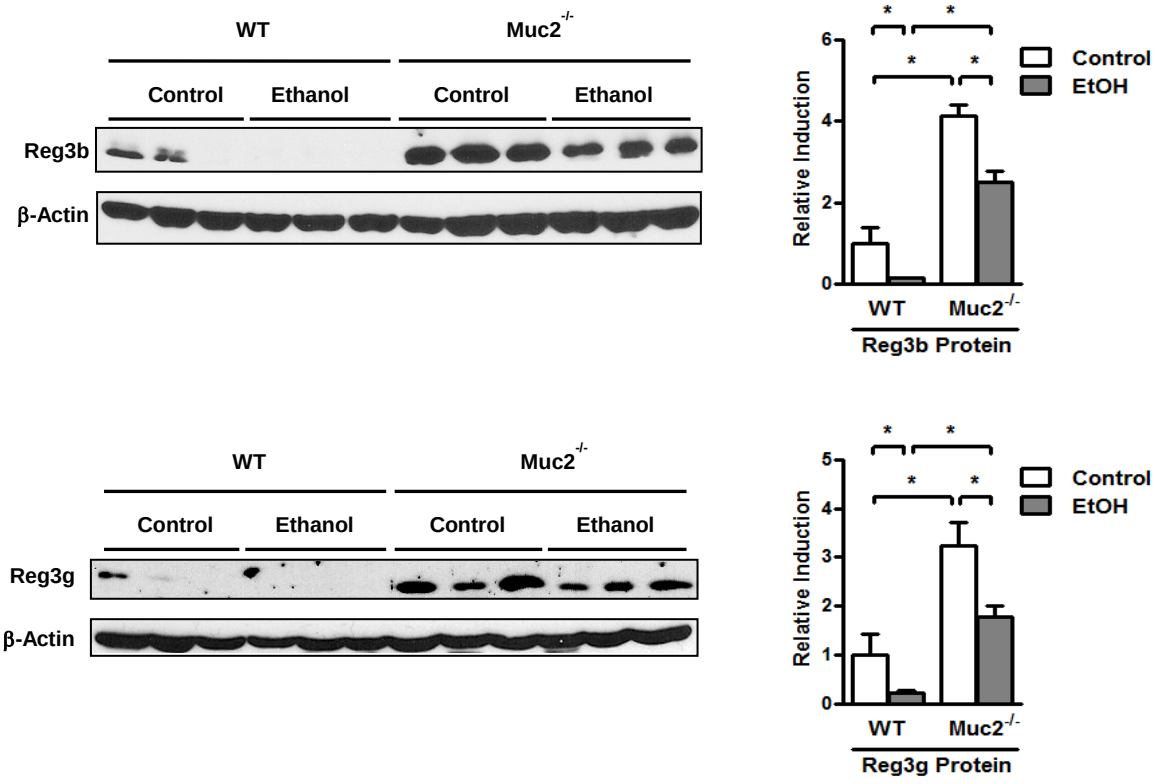
intragastrische Alkoholadministration in Wildtyp-Mäusen eine Proliferation von dem Gram-negativen Bakterium (33) *Akkermansia muciniphila* bewirkt (Abb. III D) (28). Obwohl nach einer Woche von Alkoholgabe dieser Effekt nicht festgestellt werden konnte, wurden signifikant weniger *Akkermansia muciniphila* in Muc2$^{-/-}$ Mäusen im Vergleich zu den Wildtyp-Pendants nach kontinuierlicher einwöchiger Alkoholapplikation in den Magen gemessen (Abb. III E). Das Wachstum von *Akkermansia muciniphila* hängt von dem Vorhandensein von Mukus *in vitro* ab jedoch nicht von Ethanol (Abb. III F). Somit resultiert die Abwesenheit von Muzin-2 in einer Dysbiose, die durch eine Abnahme des Gram-negativen Bakteriums *Akkermansia muciniphila* charakterisiert ist, die wahrscheinlich zu dem niedrigeren systemischen Level von Endotoxin beiträgt. Zusammenfassend sind Muc2$^{-/-}$ Mäuse gegen Alkohol-assoziierte quantitative und qualitative Veränderungen des Mikrobioms geschützt und demonstrieren niedrigere LPS-Level im Plasma.

Abbildung III – Muzin-2-defiziente Mäuse zeigen geringere LPS-Plasmaspiegel und keine intestinale bakterielle Überwucherung



Antimikrobielle Protein-Expression and -Aktivität sind gesteigert im Darm von Muzin-2-defizienten Mäusen. Einige Faktoren kontrollieren die intestinale Bakterienlast, jene sind insbesondere antimikrobielle Moleküle des Wirtes, die von epithelialen und Paneth-Zellen sezerniert werden. Das Labor von Dr. Schnabl konnte zeigen, dass die Expression von regenerating islet-derived 3 beta (Reg3b) und gamma (Reg3g) im Dünndarm von Mäusen nach Ethanoladministration vermindert ist (28). Die Inhibierung war besonders deutlich im proximalen Dünndarm, dem Ort der stärksten Proliferation von luminalen Bakterien und der höchsten intraluminalen Alkoholkonzentration (28). Wir bestätigten die Alkohol-bedingte Inhibition der Reg3b- und Reg3g-Proteinexpression im Jejunum von Wildtyp-Mäusen. Erstaunlicherweise war die Reg3b- und Reg3g-Expression signifikant höher in Muc2$^{-/-}$ Mäusen als in Wildtyp-Mäusen nach intragastrischer Gabe von Alkohol bzw. isokalorischer Nahrung (Abb. IV).

Abbildung IV – Muzin-2-defiziente Mäuse zeigen eine hochregulierte intestinale antimikrobielle Protein-Expression

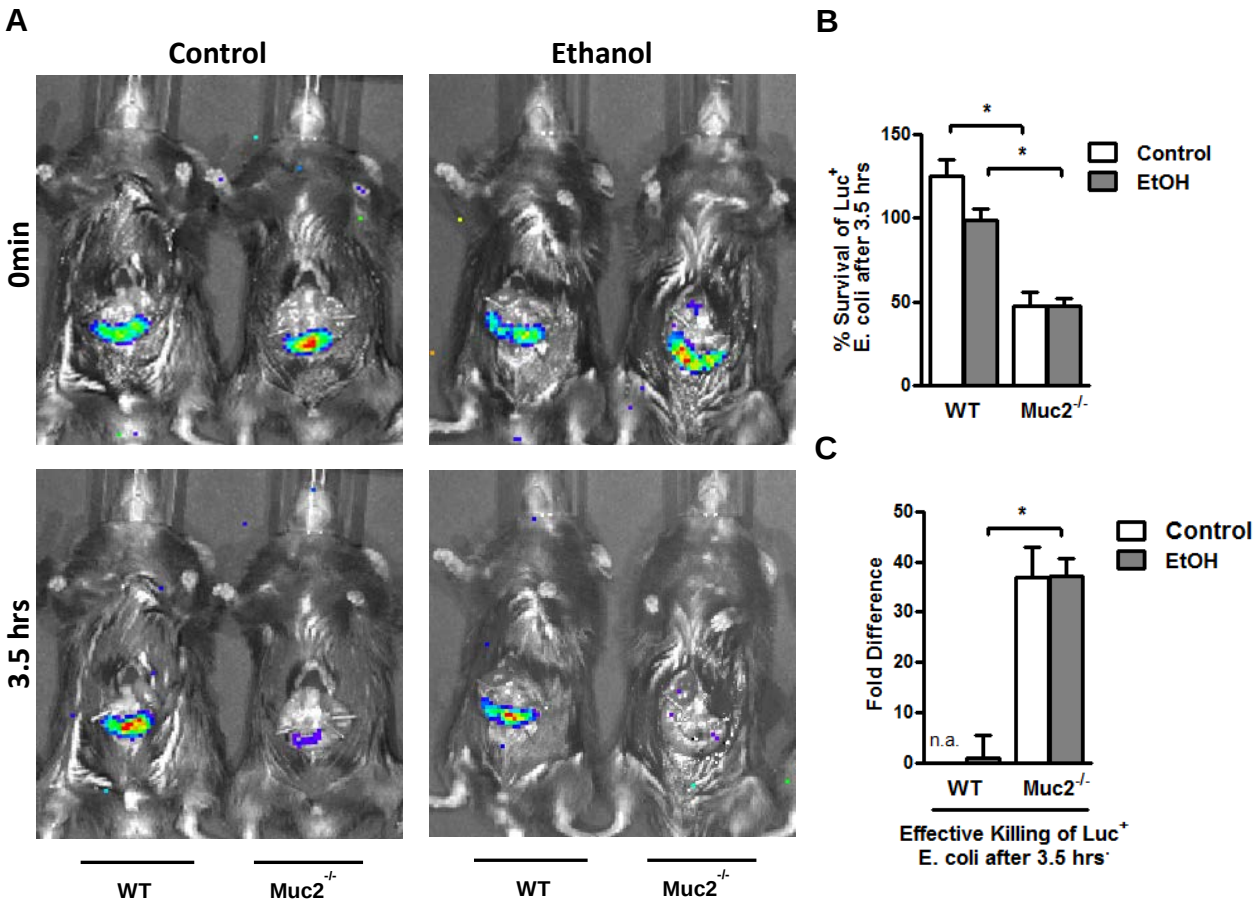


Andere antimikrobielle Moleküle wie Cathelicidin antimicrobial peptide (Camp) oder Defensin beta 1 (Defb1) zeigten ähnliche Niveaus zwischen Wildtyp- und Muc2$^{-/-}$ Mäusen nach Ethanoladministration (s. Originalartikel). Diese Ergebnisse legen nahe, dass das Fehlen von

Muzin-2 in einer starken Induktion von antimikrobiellen Faktoren mündet, die das Überleben und die Replikation der kommensalen Mikroflora beschränkt.

Um herauszufinden, ob sich diese Ergebnisse direkt in quantitative Veränderungen der kommensalen Mikroflora übersetzen lassen, haben wir ein *in vivo* luminalen Abtötungs-Assay mit nicht-pathogenem *Escherichia coli (E.coli)* im Darm von Wildtyp-Mäusen und Muzin-2-defizienten Mäusen durchgeführt (35, 36). Ein 4-cm langer Abschnitt des proximalen Jejunums wurde in anästhetisierten Mäusen ligiert (ohne Unterbrechung der Blutzirkulation) und danach wurden in diesen Abschnitt bioluminezente, nicht-pathogene *E.coli* injiziert. Zur Analyse des luminalen Überlebens und Abtötens wurde eine *In Vivo Imaging System* (IVIS)-Bildgebung der bioluminezenten *E.coli* durchgeführt zum Zeitpunkt 0 und 3,5 Stunden nach Injektion der Bakterien in den ligierten jejunalen Abschnitt.

Abbildung V – Muzin-2-defiziente Mäuse zeigen eine verstärkte intestinale antimikrobielle Aktivität



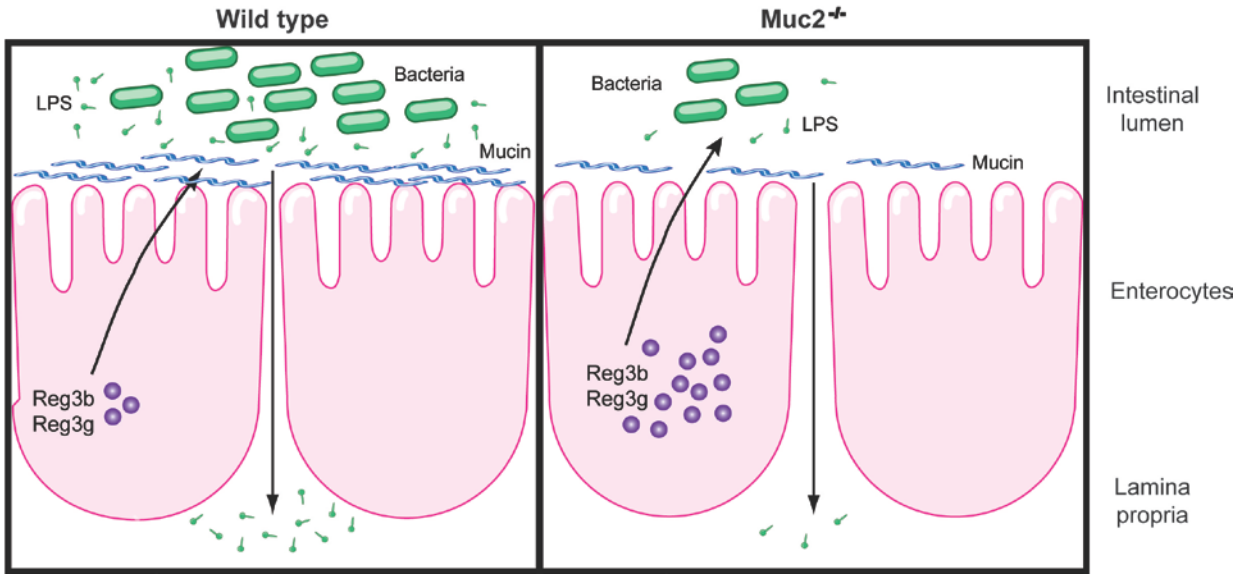
Während die ligierten Darmschlingen der Muc2$^{-/-}$ Mäuse nach Gabe einer Alkohol- bzw. isokalorischen Nahrung nach Lieber-DeCarli über zwei Wochen praktisch frei von lumineszenten Bakterien waren, wurden biolumineszente Bakterien in den jeweiligen Wildtyp-Pendants zu einem signifikant höheren Anteil nach 3,5 Stunden nachgewiesen (Abb. V A-B). Dieses Ergebnis legt nahe, dass kommensale Bakterien effektiver in jejunalen Darmschlingen von Muc2$^{-/-}$ Mäusen abgetötet werden als in Wildtyp-Mäusen (Abb. V C) und mithin die intestinale bakterielle Überwucherung nach Alkoholgabe begrenzen.

Enterale LPS-Gabe verstärkt experimentelle alkoholische Lebererkrankung in Muc2$^{-/-}$ Mäusen. Um darzulegen, dass Muc2$^{-/-}$ Mäuse geschützt sind durch Veränderungen im Verdauungstrakt und nicht durch Adaptationen in der Leber, haben wir LPS enteral verabreicht. Nach täglicher intragastrischer Gabe von LPS zusätzlich zu Ethanol über eine Woche konnten erhöhte Spiegel an bakteriellen Produkten des Gram-negativen Bakteriums *E.coli* in der Leber von Muc2$^{-/-}$ Mäusen gefunden werden, die vergleichbar waren mit denen in Wildtyp-Mäusen nach alleiniger Alkoholgabe. Die Herstellung der hepatischen Endotoxämie verschlimmerte die alkoholische Steatohepatitis in Muc2$^{-/-}$ Mäusen (Genaueres s. Originalartikel). Dies stützt unsere Feststellung, dass eine verminderte Endotoxämie zum Schutz von Muc2$^{-/-}$ Mäusen vor experimenteller alkoholischer Lebererkrankung trotz einer höheren intestinalen Permeabilität beiträgt.

Diskussion

Die erste und möglicherweise beste Möglichkeit für den Körper, die toxischen Effekte von oral aufgenommenem Alkohol zu begrenzen, ist der Verdauungstrakt. In dieser Studie untersuchten wir die Rolle von Muzinen und im Besonderen von intestinalem Muzin-2 in alkoholischer Steatohepatitis. Alkohol erhöht die Dicke der intestinalen Schleimschicht in Patienten mit Alkoholkrankheit. Alkoholische Steatohepatitis war gelindert in Muzin-2-defizienten Mäusen, was nicht durch einen veränderten Alkoholmetabolismus oder eine kompensatorische Überproduktion von anderen intestinalen Muzinen erklärt werden konnte. Wir legen dar, dass das Fehlen von Muzin-2 zu einer veränderten Zusammensetzung der Darmflora und einer verstärkten Expression von antimikrobiellen Molekülen führt. Dies ist verbunden mit einem gesteigerten intraluminalen Abtöten von Bakterien und einer Verminderung der intestinalen Bakterienlast in Muzin-2-defizienten Mäusen. Weniger bakterielle Produkte wie LPS treten vom Darm in die systemische Zirkulation über und resultieren in geringergradigem Leberschaden und weniger Steatose (Abb. VI).

Abbildung VI – Modell zur Veranschaulichung der Rolle von Muzin-2 in experimenteller alkoholischer Lebererkrankung



Experimentelle alkoholische Lebererkrankung hängt von aus dem Darm stammenden bakteriellen Produkten ab, die eine Leberschädigung und Steatose vorantreiben (2).

Veränderungen der Darmflora beeinflussen die bakterielle Translokation in Patienten und in experimenteller alkoholischer Steatohepatitis. Erhöhte Level an Endotoxin und bakterieller DNA im Plasma wurden mit bakterieller Überwucherung im Dünndarm von zirrhotischen Patienten in Verbindung gebracht. Desweiteren ist die bakterielle Überwucherung im Dünndarm ein unabhängiger und Hauptrisikofaktor für die Anwesenheit von bakterieller DNA in der systemischen Zirkulation (37, 38). Interessanterweise verminderte die selektive intestinale Dekontamination die Translokation in die mesenterischen Lymphknoten auch in nicht-zirrhotischen Patienten; obwohl dies eine noch nicht etablierte Therapie darstellt, profitieren davon auch Patienten mit alkoholischer Leberzirrhose (19, 39). Folglich prädisponiert intestinale bakterielle Überwucherung Patienten mit Leberkrankheit für bakterielle Translokation.

Das Labor von Dr. Schnabl hat bereits quantitative Veränderungen (Überwucherung) im enterischen Mikrobiom im Modell der intragastrischen Alkoholadministration in Mäusen demonstriert (28). Supprimierung von Alkohol-induzierter intestinaler bakterieller Überwucherung mit nicht-absorbierbaren Antibiotika vermindert systemische LPS-Level und verbessert alkoholische Steatohepatitis in Ratten (18). Andererseits, wenn bakterieller Überwuchs experimentell im Dünndarm geschaffen wird, führt dies zu einer Entzündung und Schädigung in der Leber (40).
Wir spekulieren, dass die Aktivierung des angeborenen Immunsystems der Schleimhaut – dargestellt durch gesteigerte Niveaus von Reg3b und Reg3g – zu einer verringerten intestinalen bakteriellen Überwucherung in $Muc2^{-/-}$ Mäusen führt. Präbiotika stellen die Expression von Reg3b und Reg3g in Wildtyp-Mäusen wieder her, limitieren die bakterielle Überwucherung und verbessern die Alkohol-induzierte Steatohepatitis (28). Nichtsdestoweniger könnten andere antimikrobielle Moleküle oder Komponenten des angeborenen Immunsystems der Schleimhaut zusammen mit Reg3b und Reg3g arbeiten, und zukünftige Studien sind erforderlich zur weiteren Untersuchung. Mithin, gestützt auf unsere Studie, schlagen wir ein Konzept vor, in welchem die Suppression von intestinaler bakterieller Überwucherung durch antimikrobielle Moleküle des Wirtes in eine verminderte Verfügbarkeit von intraluminalen bakteriellen Produkten mündet. Weniger von diesen Produkten können die intestinale Barriere in die portale Zirkulation überschreiten, was schließlich alkoholische Lebererkrankung begrenzt.

Die Forschungsgruppe um Dr. Schnabl hat darüberhinaus auch qualitative Veränderungen im enterischen Mikrobiom (Dysbiose) aufgezeigt. Ethanol-assoziierte Dysbiose ist durch eine Unterdrückung von kommensalen probiotischen Bakterien charakterisiert, einschließlich

Lactobacillus (28). Verschiedene Studien haben gezeigt, dass eine Wiederherstellung einer Eubiose mittels ergänzenden probiotischen *Lactobacillus* alkoholische Steatohepatitis in Nagetieren verbessert (41, 42). Interessanterweise sind Muc2$^{-/-}$ Mäuse geschützt vor Alkohol-bedingten Veränderungen in der mikrobiellen Zusammensetzung einschließlich einer Suppression von *Lactobacillus*. Zusätzlich begrenzt die Defizienz von Muzin-2 die Proliferation von Bakterien (wie Gram-negative *Akkermansia muciniphila*), welche Muzine als Energiequelle nutzen. Somit verhindert die Abwesenheit von Muzin-2 Alkohol-assoziierte Dysbiose, stellt die intestinale Homeostase wieder her und lindert experimentelle alkoholische Lebererkrankung.

Die Schleimschicht hat eine sehr wichtige Rolle im Darm. Sie verhindert in großem Maße das Übertreten von lebensfähigen Bakterien vom Darmlumen in extraintestinale Organe wie Lymphknoten und die systemische Zirkulation (43). Die Absenz von Muzin-2 als Hauptbestandteil der intestinalen Schleimschicht hat keinen offenbaren Nachteil für die Darm-Leber-Achse im Grundzustand ohne Lebernoxe von außen. Die Dicke der intestinalen Schleimschicht nimmt zu in Patienten mit Alkoholkrankheit – wie in unserer Arbeit dargestellt – und durch andere in Ratten nach Alkoholgabe (27), was als Verteidigungsmechanismus gegenüber Alkohol oder gegenüber einer Verletzung der intestinalen Epithelzellen interpretiert werden kann. Und tatsächlich steigern enterische Infektionen die Muzin-2-Produktion und die Dicke der Schleimschicht (43). Die Kehrseite dieser offensichtich guten Reaktion das Darmes, die Schleimschicht zu stärken, ist, dass das Immunsystem der Enterozyten gegenüber Bakterien behindert wird. Wir können zur Zeit nur spekulieren, wie sehr eine dickere Schleimschicht die Expression von antimikrobiellen Molekülen als Teil des angeborenen Immunsystems der Schleimhaut beeinflusst. Eine Möglichkeit ist, dass bakterielle Liganden den Enterozyten nicht in ausreichendem Maße zugänglich sind, um die Expression von antimikrobiellen Proteinen zu stimulieren. Es wurde gezeigt, dass die Reg3g-Expression von toll-like receptor-4 (TLR4) und Interleukin-22 abhängig ist und dass sie durch Flagellin stimuliert werden kann (34, 44, 45). Sie wird auch durch zell-autonome MyD88-abhängige TLR-Aktivierung in intestinalen Paneth-Zellen induziert (46). Folglich erhöht sich bei Provokation des Körpers durch Alkohol die Dicke der intestinalen Schleimschicht und weniger antimikrobielle Moleküle erreichen das Lumen, um die Proliferation von intestinalen Bakterien zu kontrollieren. Eine scheinbar gute Antwort des Körpers als Reaktion auf Alkohol-induzierten Epithelzellschaden behindert das angeborene Immunsystem der Schleimhaut und mündet in einem Versagen des intestinalen Homeostase-Systems. Man sollte anmerken, dass dies nicht eine generelle Antwort in Muzin-2-defizienten Mäusen ist auf eine Verletzung oder Entzündung

des Darmes hin sondern eher spezifisch für Alkohol. Andere Studien haben gezeigt, dass eine durch das pathogene Bakterium *Citrobacter rodentium* induzierte Kolitis in Muc2$^{-/-}$ Mäusen verstärkt auftritt (43).

Unsere Arbeit zeigt, dass das Fehlen von einem einzigen Wirtsgen Muzin-2 – welches nicht in der Leber oder in inflammatorischen Zellen exprimiert wird, sondern größtenteils auf den Darm begrenzt ist – alkoholische Steatohepatitis lindert. Unsere Ergebnisse verstärken das Konzept, dass experimentelle alkoholische Lebererkrankung von dem Darm abhängig ist. Mit Alkohol verbundene Veränderungen des Mikrobioms – insbesondere intestinale bakterielle Überwucherung – trägt zum Alkohol-induzierten Leberschaden bei. Alles in allem betont unsere Studie erneut die Wichtigkeit der Darm-Leber-Achse. Eine Behandlung, die auf das angeborene Immunsystem der Schleimhaut sowie auf intestinale bakterielle Überwucherung abzielt, könnte das klinische Management von Patienten mit alkoholischer Lebererkrankung erweitern.

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Deficiency of Intestinal Mucin-2 Ameliorates Experimental Alcoholic Liver Disease in Mice

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The intestinal mucus layer protects the epithelium from noxious agents, viruses, and pathogenic bacteria present in the gastrointestinal tract. It is composed of mucins, predominantly mucin (Muc) 2, secreted by goblet cells of the intestine. Experimental alcoholic liver disease requires translocation of bacterial products across the intestinal barrier into the systemic circulation, which induces an inflammatory response in the liver and contributes to steatohepatitis. We investigated the roles of the intestinal mucus layer, and in particular Muc2, in development of experimental alcohol-associated liver disease in mice. We studied experimental alcohol-induced liver disease, induced by the Tsukamoto-French method (which involves continuous intragastric feeding of an isocaloric diet or alcohol) in wild-type and $Muc2^{-/-}$ mice. $Muc2^{-/-}$ mice showed less alcohol-induced liver injury and steatosis than developed in wild-type mice. Most notably, $Muc2^{-/-}$ mice had significantly lower plasma levels of lipopolysaccharide than wild-type mice after alcohol feeding. In contrast to wild-type mice, $Muc2^{-/-}$ mice were protected from alcohol-associated microbiome changes that are dependent on intestinal mucins. The antimicrobial proteins regenerating islet-derived 3 beta and gamma were expressed at significantly higher levels in the jejunum of $Muc2^{-/-}$ mice fed the isocaloric diet or alcohol compared with wild-type mice. Consequently, $Muc2^{-/-}$ mice showed increased killing of commensal bacteria and prevented intestinal bacterial overgrowth. *Conclusion*: $Muc2^{-/-}$ mice are protected from intestinal bacterial overgrowth and dysbiosis in response to alcohol feeding. Subsequently, lower amounts of bacterial products such as endotoxin translocate into the systemic circulation, decreasing liver disease. (HEPATOLOGY 2013;00:000–000)

Liver cirrhosis is the twelfth leading cause of death in the United States, and 48% of all deaths from cirrhosis are alcohol-related.[1] Alcoholic liver disease comprises hepatic steatosis, which may progress to alcoholic hepatitis, fibrosis, and cirrhosis.[2] There is strong evidence for a gut-liver axis that is causatively related to alcohol-induced liver disease, both in patients and in experimental animal models. Gastrointestinal permeability is greater in alcoholics compared with normal subjects.[3,4] Several animal studies have demonstrated that ethanol disrupts the intestinal epithelial barrier function via a direct effect of ethanol and/or its metabolite acetaldehyde.[5] Ethanol-induced gut leakiness results in elevated plasma levels of lipopolysaccharide (LPS) or endotoxin, a major component of the gram-negative bacterial outer membrane, and subsequent liver injury.[6-8] Endotoxemia is more prevalent in patients with alcoholic

Abbreviations: Adh, alcohol dehydrogenase; Camp, cathelicidin antimicrobial peptide; Cyp2E1, cytochrome p450 enzyme 2E1; Defb1, defensin beta 1; IL-22, interleukin-22; LPS, lipopolysaccharide; Muc, mucin; Reg3, regenerating islet-derived 3.

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liver disease compared with normal subjects, and plasma endotoxin levels correlate with the severity of liver damage in patients with alcoholic hepatitis.[9-12] The most convincing evidence for a role of gut-derived endotoxin comes from mice harboring a genetic deletion in the LPS signaling pathway. Mice deficient in Toll-like receptor (TLR) 4 as the cellular LPS receptor, CD14 as the cellular co-receptor for LPS, or intracellular signaling molecules downstream of the LPS receptor are resistant to alcohol-induced liver injury.[13-15] In addition, selective intestinal decontamination with nonabsorbable antibiotics reduces plasma endotoxin levels and prevents experimental alcoholic liver disease.[16-18] Although not an established therapy, treatment with antibiotics also improves liver function in patients with alcoholic cirrhosis.[19]

The intestinal mucus layer forms a physical barrier between the underlying epithelium and the lumen of the gastrointestinal tract and protects the epithelium against noxious agents, viruses, and pathogenic bacteria. It consists of two separate sublayers: the inner layer is attached to the epithelial cell layer and is devoid of bacteria; the outer layer can be washed off easily and is colonized by bacteria.[20,21] The intestinal mucus layer is composed of mucins that are synthesized and secreted by intestinal goblet cells.[22] Two different types of mucins exist: secreted, or gel-forming mucins, and membrane-bound mucins. There are three gastrointestinal secreted mucins (Muc2, Muc5AC, and Muc6) that are characteristically large, heavily *O*-glycosylated glycoproteins assembled into oligomers that contribute to the viscous properties of intestinal mucus layer.[23] The intestinal membrane-bound mucins (Muc1, Muc3-4, Muc12-13, and Muc17) protect against pathogens that penetrate the inner mucus layer.[24] The major and most abundant secreted mucin in the small and large intestine is mucin-2.[25] Mice deficient in Muc2 are prone to colorectal cancer and appear to have a disrupted epithelial homeostasis.[25] Specific clinical symptoms such as spontaneous colitis depend on their genetic mouse strain background.[26]

It has been reported that the intestinal mucus increases after alcohol feeding in rats.[27] However, there are currently no patient data or experimental studies assessing the functional contribution of the intestinal mucus layer in alcoholic liver disease. We therefore took an unbiased approach to study the role of the intestinal mucus layer, in particular Muc2, using a mouse model of alcoholic liver injury and steatosis.

Materials and Methods

Animal Models of Alcohol Feeding. Male wild-type mice (C57BL/6J) were purchased from The Jackson Laboratory or bred in the vivarium associated with our laboratory. Male Muc2$^{-/-}$ mice (back-crossed to C57BL/6J for more than 10 generations) were kindly provided by Anna Velcich (Albert Einstein College of Medicine, Yeshiva University, New York, NY). Age-matched mice were used for this study. All animals received humane care in compliance with institutional guidelines. The intragastric feeding model of continuous ethanol infusion in mice has been described.[28]

The Lieber DeCarli diet model of alcohol feeding for 2 weeks was used to determine intestinal permeability and for an *in vivo* luminal killing assay. We opted to assess intestinal permeability in a complementary and noninvasive mouse model of alcoholic steatohepatitis using the Lieber DeCarli diet, because prior surgery and the implanted gastrostomy catheter could affect accurate assessment of intestinal permeability. To avoid two surgeries in the same mouse, we also chose to assess *in vivo* luminal killing of bacteria in mice that were fed the Lieber DeCarli diet.

Additional materials and methods are described in the Supporting Information.

Results

Alcohol Abuse Increases the Thickness of the Intestinal Mucus Layer in Humans. It has been reported that chronic alcohol feeding increases the total mucus content in the small intestine in rats.[27] We have confirmed these data in humans. Alcoholics show a significant increase in the thickness of the mucus layer on duodenal biopsies compared with healthy humans (Fig. 1A,B).

Muc2-Deficient Mice Have Decreased Alcoholic Steatohepatitis. To investigate the role of the intestinal mucus layer in experimental alcoholic liver disease,

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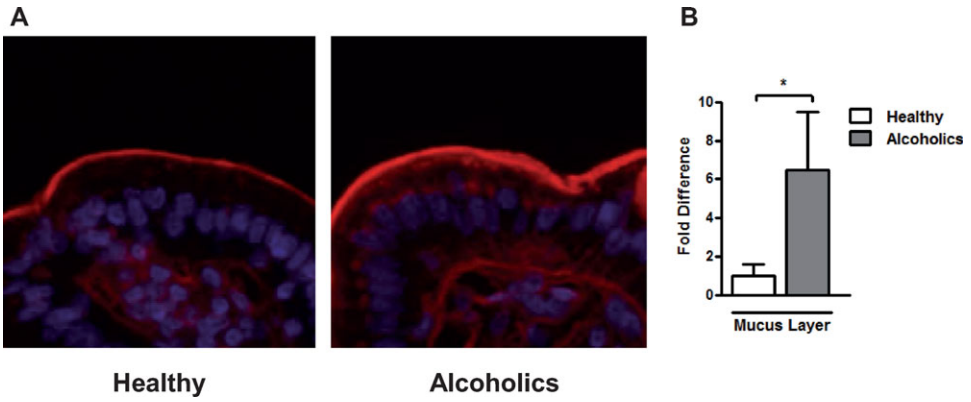

Fig. 1. Ethanol increases the intestinal mucus layer. The small intestinal mucus layer was determined on duodenal biopsies obtained from healthy controls (n = 12) and patients with chronic alcohol abuse (n = 8) using wheat germ agglutinin staining. (A) Representative intestinal sections are shown (magnification ×200). (B) Densitometry of sections was performed. *$P < 0.05$.

we used mice harboring a genetic deletion in the Muc2 gene.[25] Muc2 is the most abundant secreted mucin in the gastrointestinal tract[25] and its absence results in a significantly thinner mucus layer in mice as shown by Periodic acid–Schiff (PAS) staining of the small intestine (Fig. 4A). To confirm that Muc2 expression is largely restricted to the intestine, we measured Muc2 messenger RNA levels in several organs from wild-type mice. Muc2 gene expression was highest in the small and large intestine, but it was undetectable in the liver or bone marrow–derived cells (Supporting Fig. 1A). These findings were confirmed by immunofluorescent staining. Muc2 protein was abundantly expressed in the small intestine (Supporting Fig. 1B, left panel), but undetectable in the liver of wild-type mice (Supporting Fig. 1B, right panel). Small intestine from Muc2-deficient mice served as a negative staining control (Supporting Fig. 1B, middle panel).

We therefore subjected wild-type and $Muc2^{-/-}$ mice to the intragastric feeding model of continuous ethanol infusion for 1 week. Mice fed an isocaloric diet served as controls. Administration of ethanol lead to a comparable increase of liver weight to body weight ratio (Supporting Fig. 2A). Plasma alanine aminotransferase (ALT) levels as measures for liver injury were significantly lower in alcohol-fed $Muc2^{-/-}$ mice compared with wild-type mice (Fig. 2A). Micro- and macrovesicular steatosis occurred after 1 week following alcohol administration compared with wild-type mice receiving an isocaloric diet. Hepatic fat accumulation was markedly lower in $Muc2^{-/-}$ mice compared with wild-type mice following 1 week of continuous intragastric ethanol feeding (Fig. 2B). This was confirmed by lower hepatic triglycerides in $Muc2^{-/-}$ mice after alcohol administration (Fig. 2C). Plasma triglyceride levels were similar between wild-type and $Muc2^{-/-}$ mice fed an isocaloric and alcohol diet intragastrically for 1 week (Supporting Fig. 2B) suggesting no difference in intestinal lipid absorption. Hepatic oxidative stress was also significantly lower in $Muc2^{-/-}$ mice compared with wild-type mice following 1 week of intragastric alcohol feeding, as supported by thiobarbituric acid reactive substances (TBARS) assay (Fig. 2D) and by staining for 4-hydroxynonenal (Fig. 2E). Thus, Muc2 deficiency, and hence a thinner intestinal mucus layer, ameliorates experimental alcohol-induced steatohepatitis.

Alcohol Metabolism and Expression of Intestinal Mucins After Ethanol Feeding in Muc2-Deficient Mice. To explain the different hepatic phenotype, we investigated whether Muc2 deficiency affects the intestinal absorption or hepatic metabolism of alcohol. Plasma alcohol levels were found to be comparable in wild-type and $Muc2^{-/-}$ mice following 1 week of intragastric alcohol feeding (Fig. 3A). Alcohol dehydrogenase (Adh) and cytochrome p450 enzyme 2E1 (Cyp2E1) are the two main hepatic enzymes to metabolize alcohol and to convert alcohol to acetaldehyde.[29] Microsomal Cyp2E1 protein was similarly up-regulated in the ethanol-treated groups (Fig. 3B). Despite higher hepatic Adh activity in $Muc2^{-/-}$ mice compared with wild-type mice after intragastric administration of an isocaloric diet that was not observed after ethanol administration (Fig. 3C), plasma acetaldehyde levels were not different following 1 week of intragastric alcohol feeding (Fig. 3D). To investigate whether the absence of Muc2 results in a compensatory up-regulation of other intestinal mucins after ethanol administration, intestinal gene and protein expression of several mucins was assessed. Deficiency in Muc2 did not result in a compensatory increase in the thickness of the intestinal mucus layer following intragastric alcohol feeding (Fig. 4A). There was no significant difference in the gene expression of secreted mucin Muc6 or of membrane-bound mucins (such as Muc1 and Muc4) in $Muc2^{-/-}$ mice relative to wild-type mice after 1 week of intragastric feeding of ethanol (Fig. 4B). These findings were confirmed using immunohistochemistry for Muc1 and Muc4 in small intestinal sections of

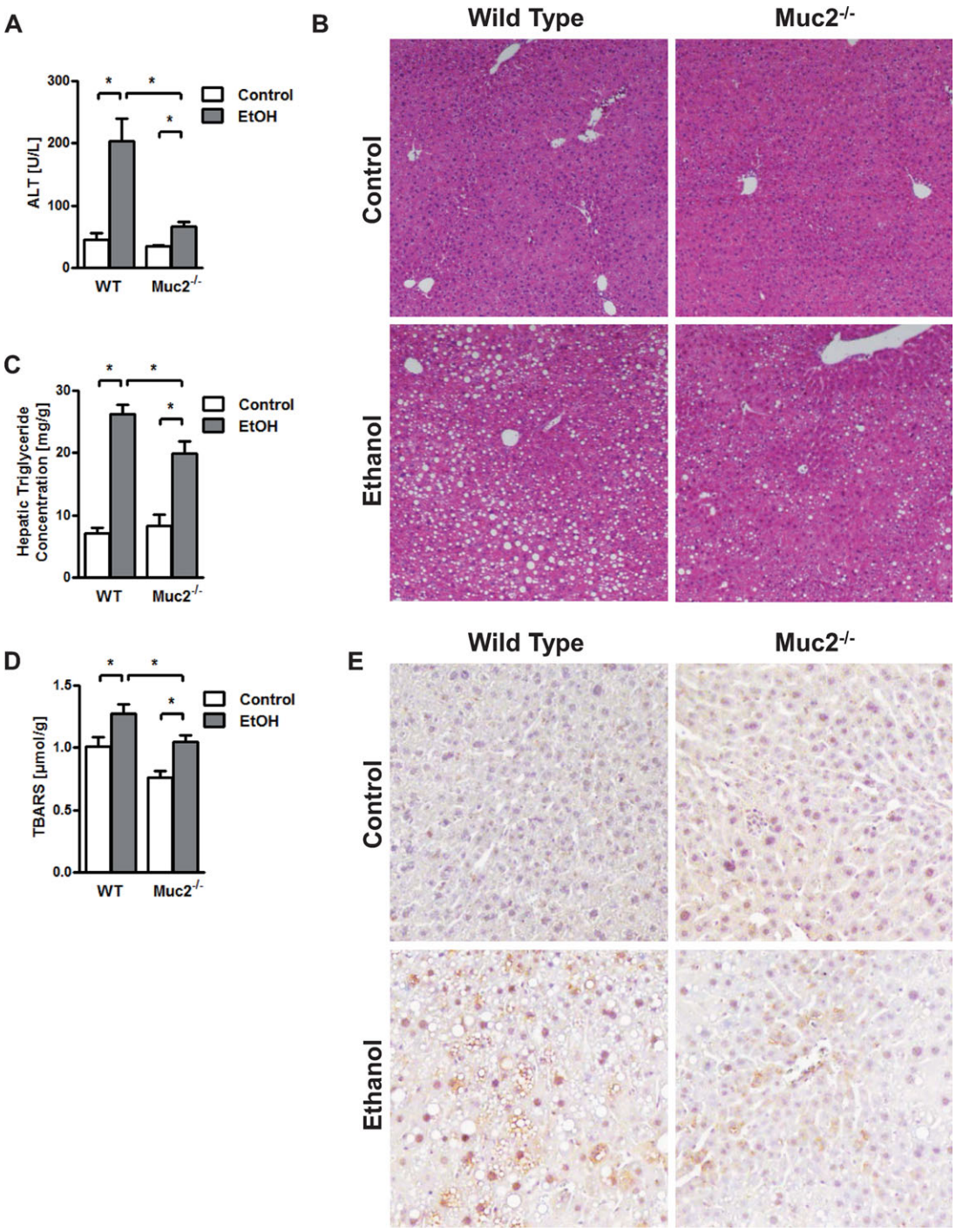

Fig. 2. Muc2 deficiency ameliorates experimental alcoholic liver injury and steatosis. Wild-type or Muc2$^{-/-}$ mice were fed an intragastric isocaloric diet (n = 5-6) or alcohol (n = 9-14) for 1 week. (A) Plasma alanine aminotransferase levels. (B) Representative liver sections after hematoxylin-eosin staining. (C) Hepatic triglyceride levels. (D) Hepatic oxidative stress levels. (E) Staining for 4-hydroxynonenal. *$P < 0.05$. Abbreviations: EtOH, ethanol; TBARS, thiobarbituric acid reactive substances; WT, wild-type.

wild-type and Muc2$^{-/-}$ mice fed an intragastric isocaloric or alcohol diet (Fig. 4C,D).

Muc2-Deficient Mice Exhibit Lower Plasma LPS Levels and Are Protected from Microbiome Changes After Alcohol Feeding. Alcoholic steatohepatitis is dependent on endotoxin derived from intestinal bacteria.[2,30] Since Muc2 is expressed in the intestine but not the liver, we next investigated whether translocation of bacterial products from the intestine to the systemic circulation is affected by the absence of Muc2. Indeed, systemic endotoxin levels were significantly lower in Muc2$^{-/-}$ mice that were fed an isocaloric diet

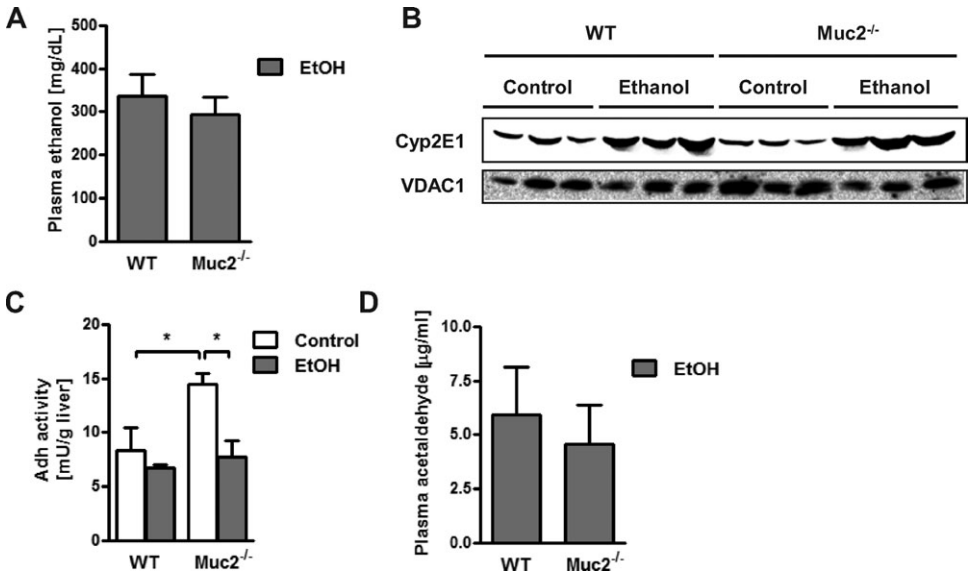

Fig. 3. Muc2-deficient mice exhibit an alcohol metabolism similar to wild-type mice after ethanol feeding. Wild-type or Muc2$^{-/-}$ mice were fed an intragastric isocaloric diet (n = 2-5) or alcohol (n = 7-12) for 1 week. (A) Plasma ethanol levels. (B) Western blot for Cyp2E1 protein in liver microsomes. Voltage-dependent anion-selective channel protein 1 (VDAC1) is shown as loading control for liver microsomes. (C) Hepatic Adh activity. (D) Plasma acetaldehyde levels. *$P < 0.05$. Abbreviations: EtOH, ethanol; WT, wild-type.

and alcohol intragastrically for 1 week compared with wild-type mice (Fig. 5A). Altered intestinal permeability or a quantitative decrease of the intestinal microflora might allow less endotoxin to escape from the gut into the systemic circulation. We therefore assessed intestinal permeability by measuring fecal albumin following a Lieber DeCarli diet for 2 weeks.[31] Fecal albumin was higher in Muc2-deficient mice at baseline and after alcohol feeding indicative of increased intestinal permeability (Fig. 5B). To confirm our findings and to directly assess intestinal permeability, we used an *in vivo* method by measuring recovery of ingested dextran labeled with fluorescein isothiocyanate. Isocaloric Lieber DeCarli diet or alcohol feeding for 2 weeks resulted in a significant increase of fluorescence in the plasma of Muc2$^{-/-}$ mice compared with wild-type mice indicative of increased intestinal permeability (Fig. 5C). Thus, despite a leakier gut barrier, Muc2$^{-/-}$ mice showed lower translocation of bacterial products.

Only a minority of the enteric bacteria can be cultured by conventional culture techniques.[32] To assess quantitative changes in the intestinal microbiome, the total bacterial load was measured by quantitative polymerase chain reaction using universal 16S ribosomal RNA bacterial primer sets. As reported by us,[28] intragastric ethanol feeding induced intestinal bacterial overgrowth in wild-type mice compared with wild-type mice fed an isocaloric diet (Fig. 5D). Interestingly, Muc2$^{-/-}$ mice are protected from intestinal bacterial overgrowth after alcohol feeding (Fig. 5D).

We have also shown that alcohol-associated changes in the enteric microbiome are characterized by a significant suppression of the commensal probiotic microflora, including *Lactobacillus*.[28] We have confirmed a significant reduction of *Lactobacillus* in wild-type mice following intragastric ethanol feeding for 1 week compared with control animals (Fig. 5E). Muc2$^{-/-}$ mice are not only protected from a suppression of *Lactobacillus*, they actually demonstrate higher numbers of *Lactobacillus* after alcohol feeding compared with control Muc2$^{-/-}$ mice (Fig. 5E). In addition, we have previously shown and confirmed that chronic intragastric alcohol feeding for 3 weeks results in an increase of Gram-negative[33] *Akkermansia muciniphila* (Fig. 5F, left panel).[28] Although no significant change was observed in wild-type mice following 1 week of intragastric alcohol feeding compared with isocaloric diet feeding, *A. muciniphila* was significantly lower in Muc2$^{-/-}$ mice compared with wild-type mice after alcohol feeding (Fig. 5F, middle panel). Growth of *A. muciniphila* is dependent on the presence of mucus *in vitro*, but not ethanol (Fig. 5F, right panel). Thus, the absence of Muc2 results in dysbiosis characterized by a decrease in gram-negative *A. muciniphila* that likely contributes to lower systemic levels of endotoxin. Littermate and nonlittermate wild-type mice did not show significant differences at baseline in alanine aminotransferase (ALT); intestinal permeability; intestinal bacterial burden; the quantity of the two major intestinal bacterial phyla, Bacteroidetes and Firmicutes;

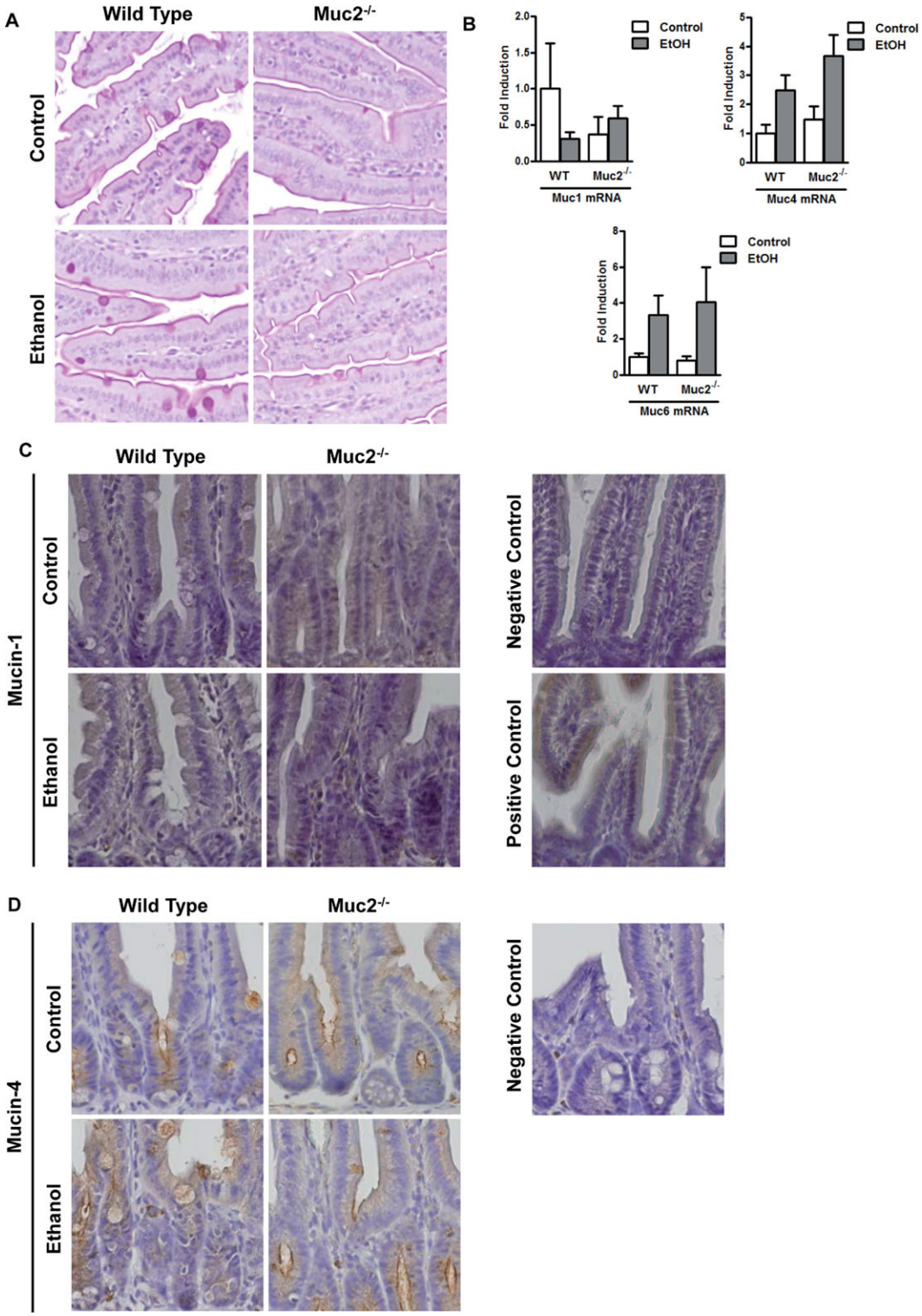

Fig. 4. Expression of intestinal mucins. Wild-type or Muc2$^{-/-}$ mice were fed an intragastric isocaloric diet (n = 4-6) or alcohol (n = 9-10) for 1 week. (A) Periodic acid–Schiff staining of jejunal sections. Representative sections are displayed. (B) Gene expression of membrane-bound mucins Muc1 and Muc4, and of secreted mucin Muc6 in jejunum of mice. (C) Immunohistochemical detection of Muc1 in the jejunum of mice following alcohol or control feeding for 1 week. The negative control was performed using isotype immunoglobulin G instead of the primary antibody. The positive control is small intestine from a 2-week-old male C57BL/6J mouse.[47] Representative sections are shown. (D) Immunohistochemical detection of Muc4 in the jejunum of mice following alcohol or control feeding for 1 week. The negative control was performed without primary antibody. Representative sections are shown. Abbreviations: EtOH, ethanol; mRNA, messenger RNA; WT, wild-type.

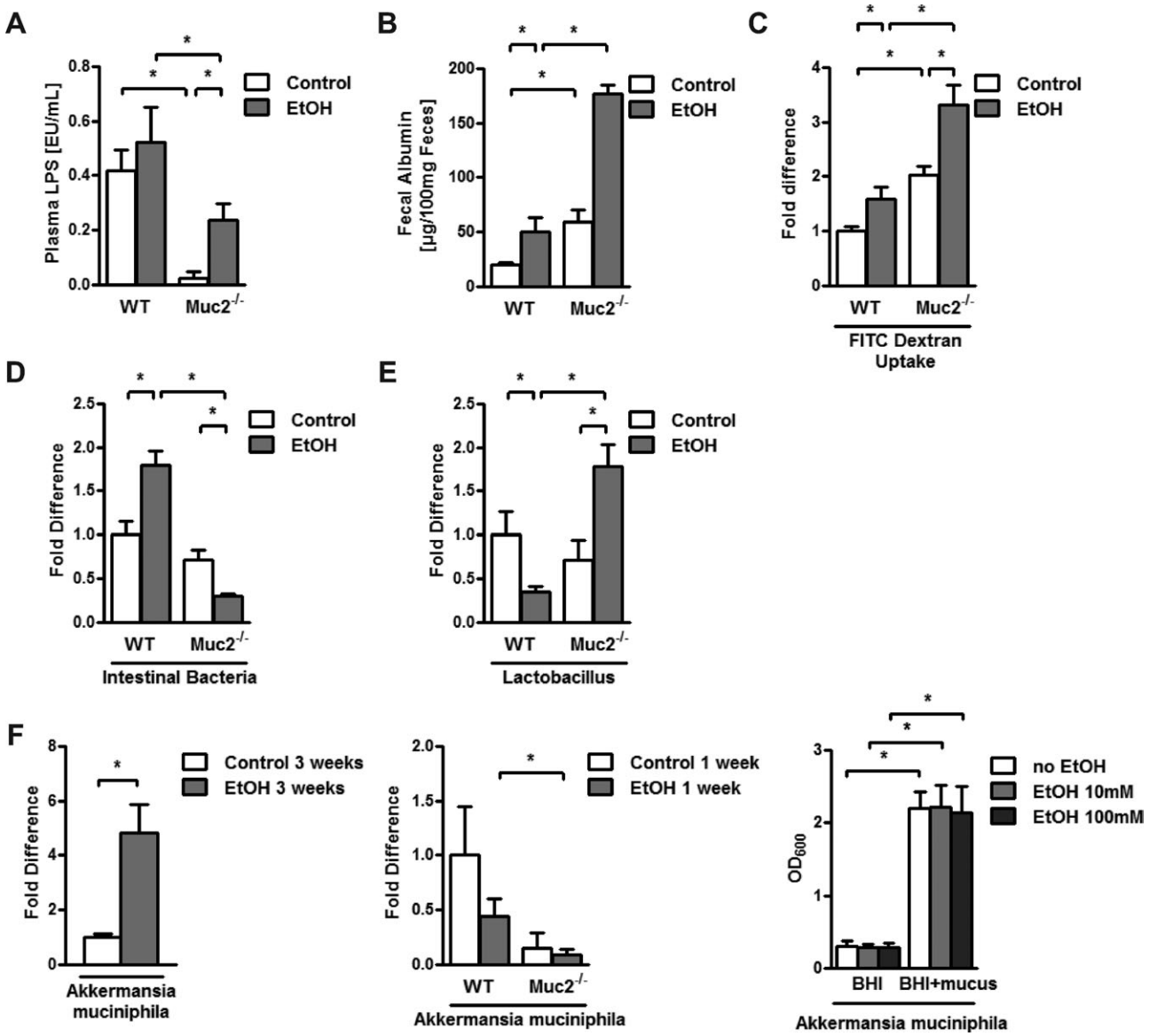

Fig. 5. Lower plasma LPS levels and no intestinal bacterial overgrowth are found in Muc2-deficient mice after alcohol feeding. Wild-type or Muc2$^{-/-}$ mice were fed an intragastric isocaloric diet (n = 4-7) or alcohol (n = 9-10) for 1 week (A, D, E, F, middle panel) or 3 weeks (F, left panel; n = 3 for isocaloric diet and n = 5 for alcohol). Mice were orally fed a Lieber DeCarli isocaloric diet (n = 3-8) or alcohol (n = 4-6) for 2 weeks (B and C). (A) Plasma endotoxin levels. (B) Fecal albumin content. (C) Fluorescein isothiocyanate–dextran in plasma 4 hours after gavage. (D, E, F, middle panel) Total intestinal bacteria, *Lactobacillus* and *A. muciniphila* in cecum of mice. (F, left panel) *Akkermansia muciniphila* in the small intestine of mice. (F, right panel) *A. muciniphila* was cultured *in vitro* in brain heart infusion (BHI) medium with or without mucus in the absence or presence of 10 or 100 mM ethanol for 24 hours. OD600 was measured. The data shown represent the mean of three independent experiments (n = 4-6). *$P < 0.05$. Abbreviations: EtOH, ethanol; WT, wild-type.

and *Lactobacillus* (Supporting Fig. 3). Taken together, Muc2$^{-/-}$ mice are protected from alcohol-associated quantitative and qualitative changes in the microbiome and have lower plasma levels of LPS.

Antimicrobial Protein Expression and Activity Are Enhanced in the Intestine of Muc2-Deficient Mice. Several factors control the bacterial load of intestine including host antimicrobial molecules that are secreted by epithelial cells and Paneth cells. We have previously reported that the expression of regenerating islet-derived 3 beta (Reg3b) and gamma (Reg3g) are reduced in the small intestine of mice fed alcohol compared with control mice.[28] The inhibition was pronounced in the proximal small intestine, the site with the largest relative increase in luminal bacteria and the highest intraluminal alcohol concentrations.[28] We confirmed alcohol-induced inhibition of Reg3b and Reg3g protein expression in the jejunum of wild-type mice (Fig. 6A,C). Strikingly, Reg3b and Reg3g expression was much higher in Muc2$^{-/-}$ mice

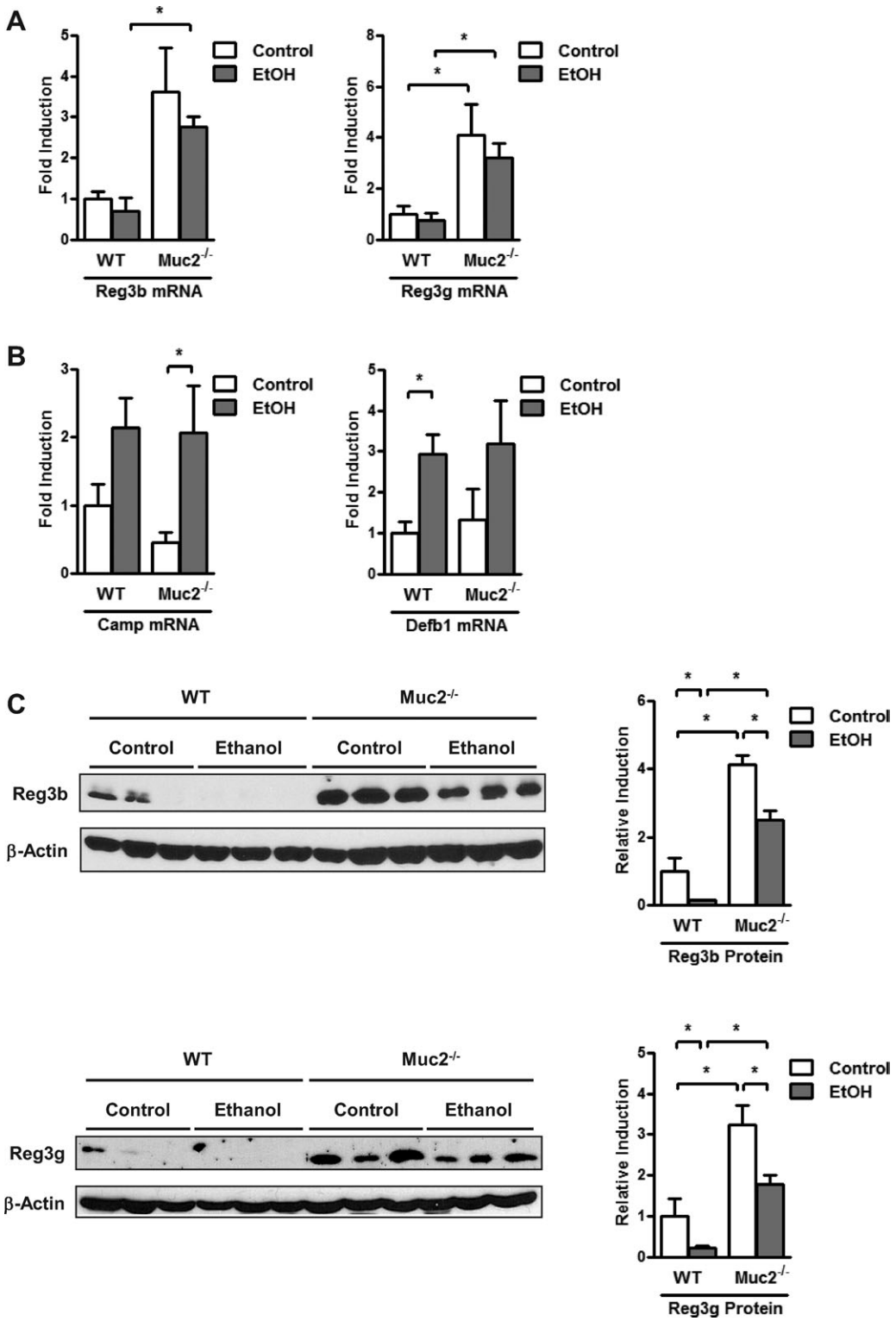

Fig. 6. Muc2-deficient mice display an up-regulated intestinal antimicrobial protein expression. Wild-type or Muc2$^{-/-}$ mice were fed an intragastric isocaloric diet (n = 4-6) or alcohol (n = 9-10) for 1 week. (A, B) Gene expression of Reg3b, Reg3g, Camp, and Defb1 in jejunum of mice. (C) Western blot for Reg3b and Reg3g. β-Actin was used as a loading control. Representative western blots are shown, which were reproduced (n = 6 in each group). Densitometry of western blot images was performed. *$P < 0.05$. Abbreviations: EtOH, ethanol; mRNA, messenger RNA; WT, wild-type.

receiving an isocaloric diet or alcohol via an intragastric feeding tube for 1 week compared with wild-type mice (Fig. 6A,C). Other antimicrobial molecules such as cathelicidin antimicrobial peptide (Camp) or defensin beta 1 (Defb1) show similar responses to intragastric alcohol in wild-type and Muc2$^{-/-}$ mice (Fig. 6B).

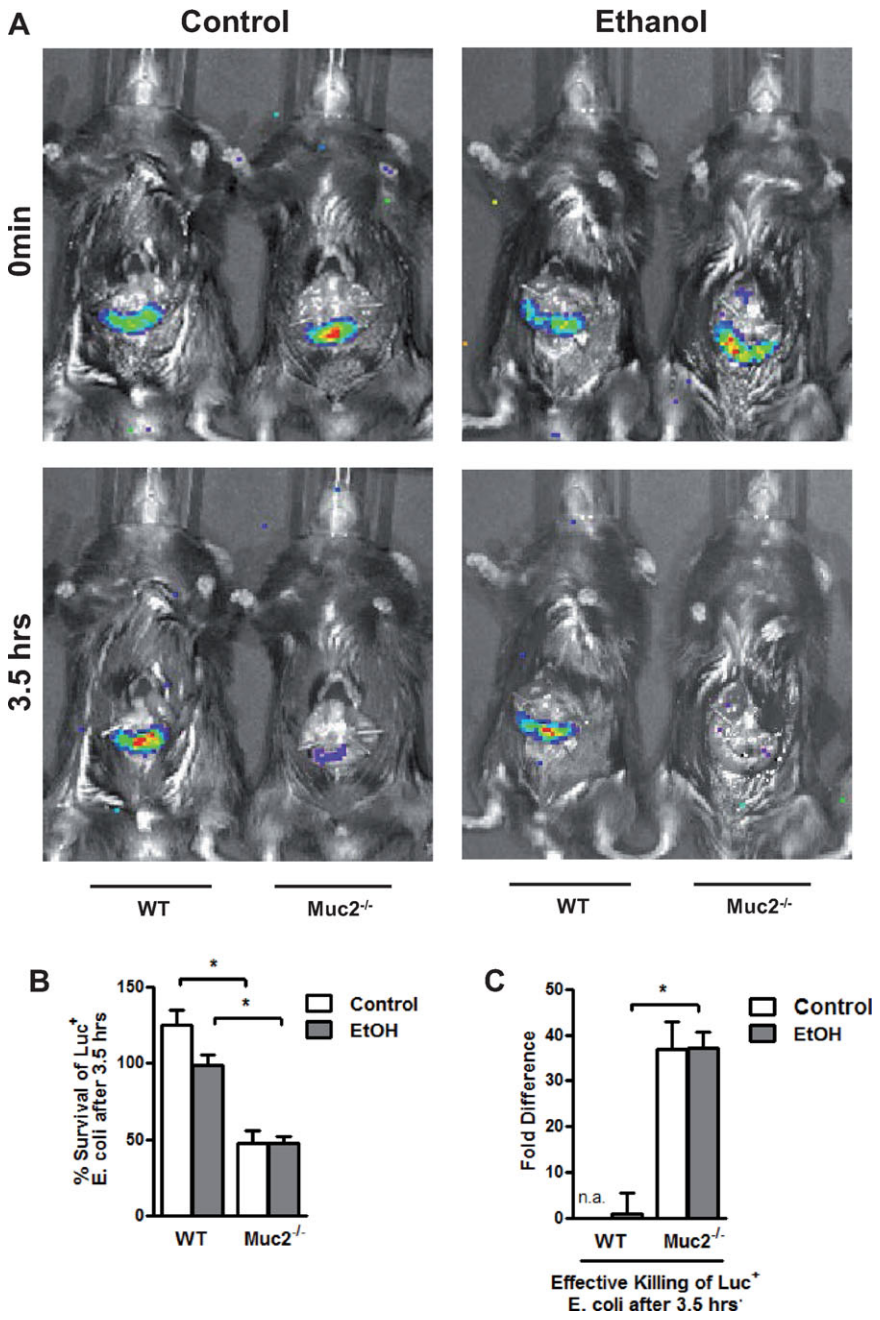

Fig. 7. Muc2-deficient mice exhibit an enhanced intestinal antimicrobial activity. Wild-type or Muc2$^{-/-}$ mice were orally fed a Lieber DeCarli isocaloric diet (n = 3-7) or alcohol (n = 4-6) for 2 weeks. (A) Bioluminescent imaging after injection of luciferase-expressing *E. coli* into jejunal loop at 0 minutes and 3.5 hours. (B, C) Survival in percentage (B) and effective killing (C) of injected *E. coli* after 3.5 hours. *$P < 0.05$. Abbreviations: EtOH, ethanol; WT, wild-type.

Interleukin-22 (IL-22) is required for the induction of intestinal Reg3b and Reg3g expression.[34] IL-22 gene expression showed a trend to be higher expressed in the small intestine of isocaloric and ethanol-fed Muc2$^{-/-}$ mice compared with wild-type mice (Supporting Fig. 4). These results suggest that Muc2 deficiency results in a strong induction of antimicrobial factors that restrict survival or replication of the commensal microflora.

To investigate whether these findings directly translate into quantitative alterations of the commensal microflora, we used an *in vivo* luminal killing assay of nonpathogenic *Escherichia coli* in the gut of wild-type and Muc2-deficient mice as described by us.[35,36] A 4-cm loop of the proximal jejunum was ligated (without interrupting the blood supply) in anesthetized mice and injected with bioluminescent, nonpathogenic *E. coli*. To analyze luminal survival and killing, IVIS imaging of bioluminescent *E. coli* was performed at 0 minutes and 3.5 hours after injection of bacteria into ligated jejunal loops. Whereas loops of Muc2$^{-/-}$ mice after feeding a Lieber DeCarli isocaloric diet or alcohol for 2 weeks were essentially devoid of luminescent bacteria, bioluminescent bacteria were found in alcohol and control fed wild-type mice at a significantly higher percentage after 3.5 hours (Fig. 7A,B). This result

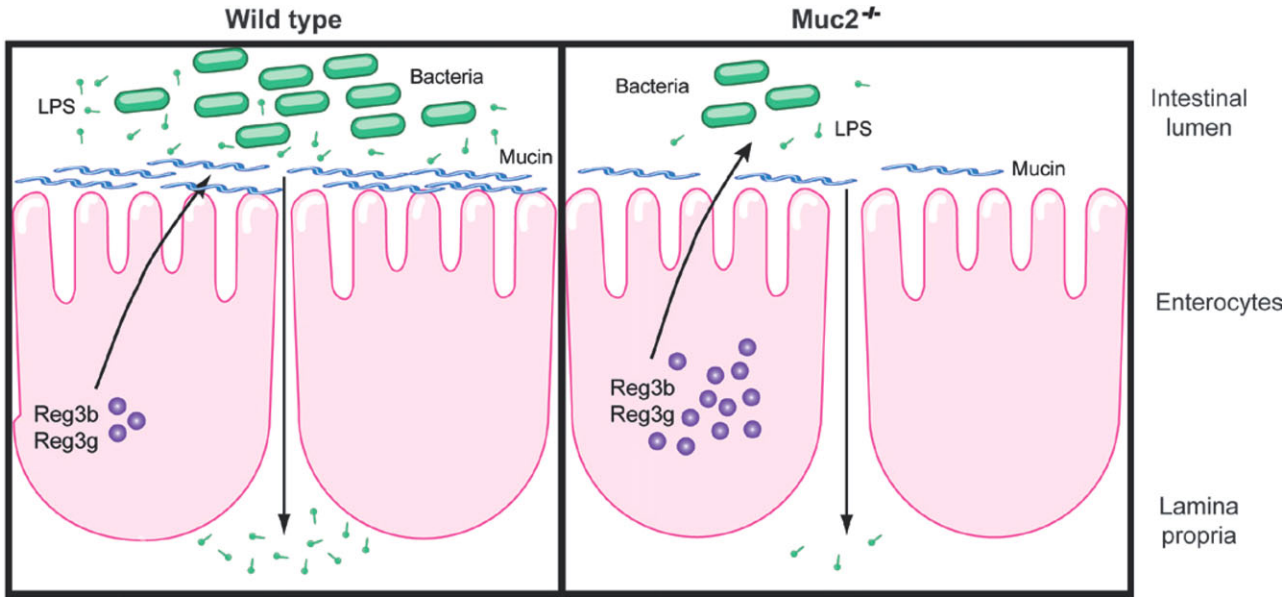

Fig. 8. Model depicting the role of Muc2 in experimental alcoholic liver disease.

suggests that commensal bacteria are killed more effectively in jejunal loops of Muc2$^{-/-}$ mice than in wild-type mice (Fig. 7C), thereby limiting intestinal bacterial overgrowth after alcohol feeding.

Enteral LPS Administration Increases Experimental Alcoholic Liver Disease in Muc2$^{-/-}$ Mice. To demonstrate that Muc2$^{-/-}$ mice are protected due to intestinal changes, but not secondary to hepatic adaptations, we have chosen to administer LPS enterally. When mice were given LPS through the intragastric feeding tube daily for 1 week in addition to ethanol, increased bacterial products from gram-negative *E. coli* were found in the livers of Muc2$^{-/-}$ mice comparable to levels seen in wild-type mice (Supporting Fig. 5A). This restoration of hepatic endotoxemia exacerbated alcoholic steatohepatitis in Muc2$^{-/-}$ mice fed ethanol and LPS (Supporting Fig. 5B,C). This supports our finding that a decreased endotoxemia contributes to the protection of Muc2$^{-/-}$ mice from experimental alcoholic liver disease despite a leakier gut.

Discussion

The first, and arguably best, opportunity for the body to limit toxic effects of orally administered alcohol is the gastrointestinal tract. In this study, we investigated the role of mucins and in particular intestinal Muc2 in alcoholic steatohepatitis. Alcohol increases the thickness of the intestinal mucus layer in patients with alcohol abuse. Alcoholic steatohepatitis was ameliorated in mice deficient in Muc2, which could not be explained by altered ethanol metabolism or a compensatory up-regulation of other intestinal mucins. We provide evidence that Muc2 deficiency results in altered microbiome composition and an increased expression of antimicrobial molecules. This is associated with enhanced intraluminal killing of bacteria and a decrease in the intestinal bacterial burden in Muc2-deficient mice. Less bacterial products such as LPS translocate from the intestine to the systemic circulation and cause less liver injury and steatosis (Fig. 8).

Experimental alcoholic liver disease is dependent on gut-derived bacterial products that drive liver injury and steatosis.[2] There is an evolving concept that changes in the gut microflora and microbiome affect bacterial translocation, both in patients and in experimental models of alcoholic steatohepatitis. Increased plasma endotoxin and bacterial DNA have been associated with small intestinal bacterial overgrowth in patients with cirrhosis. Furthermore, small intestinal bacterial overgrowth was an independent and major risk factor for the presence of bacterial DNA in the systemic circulation in patients with cirrhosis.[37,38] Interestingly, selective intestinal decontamination decreased translocation to the mesenteric lymph nodes to the level of patients without cirrhosis, and although not an established therapy, it also benefits patients with alcoholic liver cirrhosis by improving their liver function.[19,39] Thus, intestinal bacterial overgrowth predisposes patients with liver disease to bacterial translocation.

We have recently demonstrated quantitative (overgrowth) changes in the enteric microbiome using a model of intragastric alcohol feeding in mice. Suppression of alcohol-induced intestinal bacterial overgrowth with nonabsorbable antibiotics decreases systemic levels

of LPS and ameliorates alcoholic steatohepatitis in rats.[18] On the other hand, if bacterial overgrowth is induced experimentally in the small intestine, this causes liver inflammation and injury.[40]

We speculate that activation of the mucosal innate immune system, as demonstrated by increased levels of Reg3b and Reg3g, contributes to reduced intestinal bacterial overgrowth in Muc2$^{-/-}$ mice. Prebiotics restore Reg3b and Reg3g expression, limit bacterial overgrowth and ameliorate alcohol-induced steatohepatitis.[28] However, other antimicrobial molecules or components of the mucosal innate immune system might work in concert with Reg3b and Reg3g, and future studies are required for further investigations. Thus, based on our study, we propose a concept in which suppression of intestinal bacterial overgrowth by host antimicrobial molecules results in a decreased availability of intraluminal bacterial products. Less of these products are able to cross the intestinal barrier into the portal circulation, which eventually limits alcoholic liver disease.

We have also recently demonstrated qualitative changes in the enteric microbiome (dysbiosis) using a model of intragastric alcohol feeding in mice. Alcohol-associated dysbiosis is characterized by a profound suppression of commensal probiotic bacteria, including *Lactobacillus*.[28] Several studies have shown that a restoration of eubiosis using supplemental probiotic *Lactobacillus* ameliorates alcoholic steatohepatitis in rodents.[41,42] Interestingly, Muc2$^{-/-}$ mice are protected from alcohol-associated changes in the microbial composition, including a suppression of *Lactobacillus*. In addition, Muc2 deficiency limits the proliferation of bacteria (such as gram-negative *A. muciniphila*) that use mucins as carbon source. Thus, the absence of Muc2 prevents alcohol-associated dysbiosis, restores intestinal homeostasis, and inhibits experimental alcoholic liver disease.

The mucus layer has a very important role in the intestine. It largely prevents the translocation of viable bacteria from the gut lumen to extraintestinal organs such as lymph nodes and the systemic circulation.[43] The absence of Muc2 as a major component of the intestinal mucus layer has no obvious adverse effect for the gut-liver axis at baseline without challenge. The thickness of the mucus layer increases in alcoholics as shown in our study and by others in rodents,[27] which could be interpreted as a defense against alcohol or more likely against intestinal epithelial cell injury. And indeed, enteric infections also increase the Muc2 production and the mucus layer.[43] A downside of this obvious good reaction of the intestine of increasing the mucus layer is that the vigorous immune defense system of enterocytes against bacteria is impaired. We currently can only speculate how an increase in the mucus layer might affect the expression of antimicrobial molecules as part of the mucosal innate immune system. One possibility is that bacterial ligands are not as accessible to enterocytes to stimulate the expression of antimicrobials. Reg3g expression has been shown to be TLR5 and IL-22–dependent and can be induced by flagellin,[34,44,45] but intestinal IL-22 did not correlate with Reg3 protein expression in our study. Indeed, Reg3g expression is induced through cell-autonomous MyD88-dependent TLR activation in intestinal Paneth cells.[46] Thus, when the body is challenged with alcohol, the thickness of the intestinal mucus layer increases, and less antimicrobial molecules reach the lumen to control proliferation of intestinal bacteria. An apparently good reaction of the body to respond to alcohol-induced epithelial cell damage impairs the mucosal innate immune system and results in the intestinal homeostasis system to fail. One should note that this is not a general response in Muc2-deficient mice upon intestinal injury or inflammation, but is rather specific for alcohol. Other studies have shown that colitis induced by the pathogen *Citrobacter rodentium* is exacerbated in Muc2-deficient mice.[43]

Our study demonstrates that deficiency of one host gene Muc2 that is not expressed in the liver or in inflammatory cells, but largely restricted to the intestine, decreases alcoholic steatohepatitis. Our findings are consistent with the large body of evidence that experimental alcoholic liver disease is driven by the gut. Alcohol-associated changes in the microbiome, and in particular intestinal bacterial overgrowth, contributes to alcohol-induced liver injury. Taken together, our study emphasizes again the importance of the gut-liver axis. Treatment targeting the mucosal innate immune system and intestinal bacterial overgrowth might contribute to the clinical management of alcohol-induced liver disease.

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Supplementary Materials and Methods

Supplementary Methods

Animal models of alcohol feeding. For the intragastric feeding model of continuous ethanol infusion, mice were anesthetized by injection of ketamine and xylazine, and underwent surgical implantation of a long-term gastrostomy catheter made of Tygon and silastic tubings with Dacron felt under sterile conditions. The use of a swivel allows free movement of the mouse in a micro-isolator cage. After one week acclimatization period with infusion of a control high fat diet, ethanol infusion was carried out at a dose of 22.7g/kg/day for days 1 and 2, 24.3 g/kg/day for days 3 and 4, 26 g/kg/day for days 5 and 6, and 27.5 g/kg for day 7. The ethanol dose for mice alcohol fed for three weeks increased to 29.2 g/kg/day for days 9-14 and 30.9 g/kg/day for week 3. At the initial ethanol dose, total caloric intake is set at 533 Cal/kg and the caloric percentages of ethanol, dietary carbohydrate (dextrose), protein (lactalbumin hydrolysate) and fat (corn oil) are 29%, 13%, 23%, and 35%, respectively. Vitamin, salt, and trace mineral mix are included at the recommended amounts by the Committee on Animal Nutrition of the National Research Council (AIN-76A, 4.42 g/L and 15.4 g/L, respectively, Dyets Inc, PA). Mice had access to water ad libitum (1).

The Lieber DeCarli diet consisted of administering Micro Stabilized Rod Liq AC IRR (LD101A) and Maltodextrin IRR (9598) from TestDiet and 200 Proof Ethanol from Gold Shield in a specific combination following the manufacturer's feeding directions for two weeks. In brief, the caloric intake from ethanol was 0% on day 1, 10% on day 2 and 3, 20% on day 4 and 5, 30% on day 6 and 7, and 36% from day 8 on. The Lieber DeCarli isocaloric control diet consisted of Micro Stabilized Rod Liq Diet IRR (LD101) from TestDiet following the manufacturer's feeding directions.

For enteral endotoxin administration, LPS (Sigma) was administered via the gastrostomy catheter daily for 7 days. LPS was initiated with a dose 0.15 mg on the first day and increased to 0.3 mg on the second day. From day 3 to 7, 0.6 mg was given daily via the gastric feeding tube.

Human samples. Detailed selection criteria for the patient population has been published (1, 2). Written informed consent was obtained from all patients and healthy controls. The study protocol was approved by the Ethics Committee of the Université Catholique de Louvain, in Brussels, Belgium. To preserve the mucus layer, duodenal biopsies obtained during an upper

endoscopy were fixed in Carnoy's fixative consisting of 60% Ethanol, 30% Chloroform, and 10% Glacial acetic acid for 1h. To visualize the intestinal mucus layer in humans, we used wheat germ agglutinin (WGA; Invitrogen) staining that detects N-acetyl-D-glucosamine and N-acetyl-D-neuraminic acid residues (3) from the mucins. WGA was purchased as fluorescent coupled reagent. This fluorescence-based method allowed us to more accurately determine quantitative differences between samples as compared to Periodic Acid/Schiff (PAS) staining. WGA was diluted 1:500 and samples were incubated for 10min. The stained area of the entire mucosal biopsy was captured using low power magnification and quantitated using NIH ImageJ analysis.

Intestinal permeability assays. Intestinal permeability was assessed by gastric gavage of fluorescein isothiocyanate-dextran (FITC-dextran) (4kDa; Sigma), a non-metabolizable macromolecule that is used as a permeability probe as described by us (4). All mice were gavaged with 200μL FITC-dextran (100mg/mL) 4 hrs before sacrifice. FITC-dextran measurements were performed in plasma by fluorometry. The intestinal permeability was assessed additionally by measuring the albumin content in the feces by ELISA (Bethyl Laboratories). Feces was diluted in dilution buffer (100mg/ml) and analyzed following the manufacturer's instructions.

Plasma assays. Determination of plasma alcohol and acetaldehyde levels was performed using the Ethanol Assay Kit (BioVision) and the Acetaldehyde Assay Procedure (Megazyme), respectively, following the manufacturer's protocol. All material used for harvesting blood and measuring endotoxin was pyrogen free. An endpoint chromogenic Limulus amebocyte lysate (LAL) endotoxin kit (Lonza) was used according to the manufacturer's protocol. Alanine aminotransferase (ALT) levels were measured using Infinity ALT kit (Thermo Scientific) according to the manufacturer's instructions. Plasma triglycerides were determined using the Triglyceride Quantification Kit (BioVision).

Realtime-PCR analysis. RNA was extracted from mouse tissue using Trizol (Invitrogen). RNA was digested with DNase using the DNA-free kit (Ambion) and reverse transcribed using the High Capacity cDNA Reverse Transcription kit (ABI). Gene expression was performed with Sybr Green as described (5) using primer sets specific for Muc1, Muc2, Muc4, Muc6, Reg3b, Reg3g, Camp, Defb1, and 18S gene (all from NIH qPrimerDepot). To quantify intestinal bacteria, DNA was extracted from adherent and luminal intestinal contents as described (1). DNA was amplified using published 16S rRNA primer sets for universal bacteria (6) and Sybr Green. To

determine the total bacterial load present in the cecum, the qPCR value for each sample was multiplied by the total amount of DNA per gram of cecal feces. *Lactobacillus* and *Akkermansia muciniphila* was amplified using published primer sets (7, 8) and Sybr Green.

***Akkermansia muciniphila* culture.** *A. muciniphila* (ATTC BAA-835) was previously isolated from a fecal sample from a healthy individual (9). 2×10^8 colony-forming units were inoculated in Anaerobic BHI (Difco) medium with or without supplementation of 0.25% (vol/vol) porcine mucine (Type III Sigma). Ethanol (0, 10 and 100mM) was added to the culture medium. Bacteria were grown anaerobically at 37°C for 24hrs. Bacterial growth was assessed by measuring the OD600 from each individual sample.

Protein expression analysis. Microsomes from mouse liver were isolated as described (10). Western blot analysis was performed as described (1) using anti-Reg3g (kindly provided by Dr. Lora Hooper, UT Southwestern), anti-Reg3b (R&D Systems), anti-Cyp2E1 (Millipore Corporation), anti-*Escherichia coli* (Dako), anti-VDAC1 (Abcam) as loading control for microsomal extracts, anti-beta-actin (Sigma-Aldrich) or anti-tubulin (Santa Cruz) antibodies as loading control for hepatic and intestinal samples. Densitometry of Western blot analysis was performed using NIH Image J.

Alcohol dehydrogenase (ADH) activity. Hepatic ADH activity was measured using the Alcohol Dehydrogenase Activity Assay Kit (BioVision) following the manufacturer's protocol.

Staining procedures. Formalin-fixed liver samples were embedded in paraffin and stained with hematoxylin-eosin (Surgipath), or with 4-hydroxynonenal (HNE; Alpha Diagnostic Intl. Inc.) to assess lipid peroxidation. Isotype IgG was used as a negative staining control in the latter, which did not result in a positive staining. Formalin-fixed and paraffin-embedded liver and intestinal samples were deparaffinized, rehydrated and incubated with anti-Muc2 (Santa Cruz) overnight, then incubated with an Alexa fluor 568nm-conjugated secondary antibody and imaged with fluorescent microscopy. Control sections were stained with isotype antibody and showed no staining. Immunohistochemistry was performed on formalin-fixed and paraffin-embedded jejunal sections using anti-mucin-1 (Invitrogen) or anti-mucin-4 (Abcam) antibodies. Negative controls were performed with an isotype IgG control antibody or without the primary antibody.

To preserve the mucus layer, intestines were not flushed after harvesting and fixed in Carnoy's fixative for 1h. To visualize the intestinal mucus layer in mice, we used Periodic Acid/Schiff (PAS) staining (Sigma), a commonly used chemical reagent to stain vicinal diols on sugar residues of mucins (11). PAS was performed according to the manufacturer's instructions.

Hepatic triglyceride measurement. Hepatic triglyceride levels were measured using the Triglyceride Liquid Reagents Kit (Pointe Scientific) according to the manufacturer's instructions.

***In vivo* luminal killing assay.** Non-pathogenic *Escherichia coli (E.coli)* were transfected with the pXen13 plasmid (Caliper), which is a vector carrying the original *Photorhabdus luminescens* luxCDABE operon for engineering bioluminescent bacteria. To assess the killing and survival rate of non-pathogenic and bioluminescent *E.coli in vivo*, we used an intestinal loop model as we have previously described (12, 13). After anesthesia, a midline laparotomy incision was made. An approximate 4cm long segment of the jejunum was created with two vascular hemoclips without disrupting the mesenteric vascular arcades. The length of intestine between the two clips was injected with 4.3-6x10^5 CFUs. Bioluminescence imaging was performed using IVIS Spectrum (Caliper) per instructions of the manufacturer. Mice were kept anaesthetized and bioluminescence was recorded 0 and 3.5 hrs after injection of luminescent *E.coli.*

Thiobarbituric Acid Reactive Substances (TBARS) assay. Hepatic lipid peroxidation was quantified by TBARS formation using the Oxiselect TBARS Assay Kit (Cell Biolabs) following the manufacturer's instructions.

Statistical analysis. Mann-Whitney rank sum test was used for statistical analysis. Data are presented as mean ± SEM. $P<0.05$ was selected as the level of significance.

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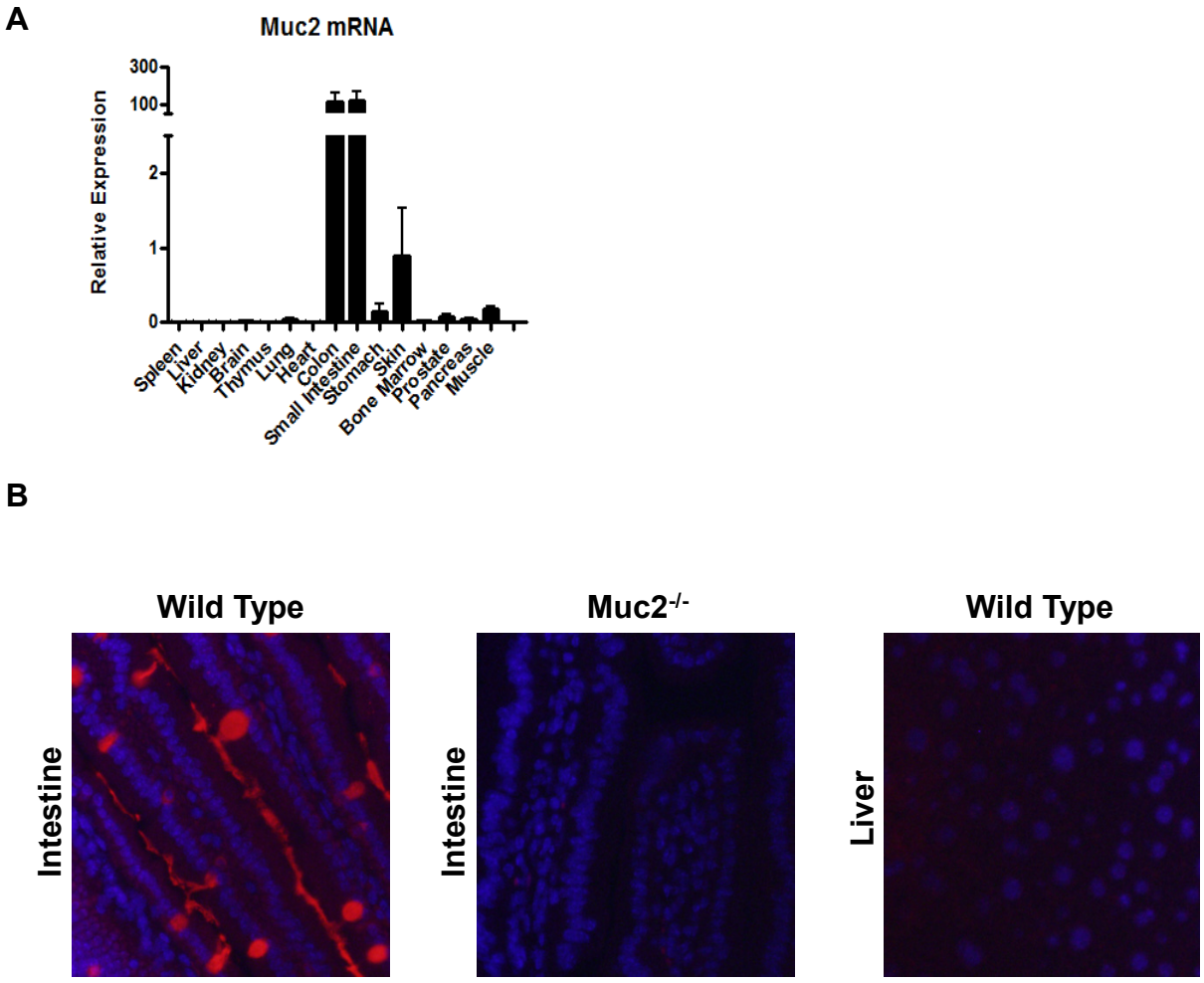
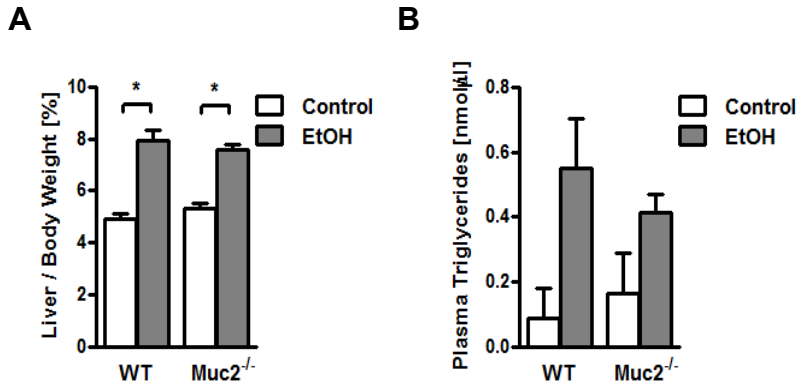
Supplementary Figures

Supplementary Figure 1



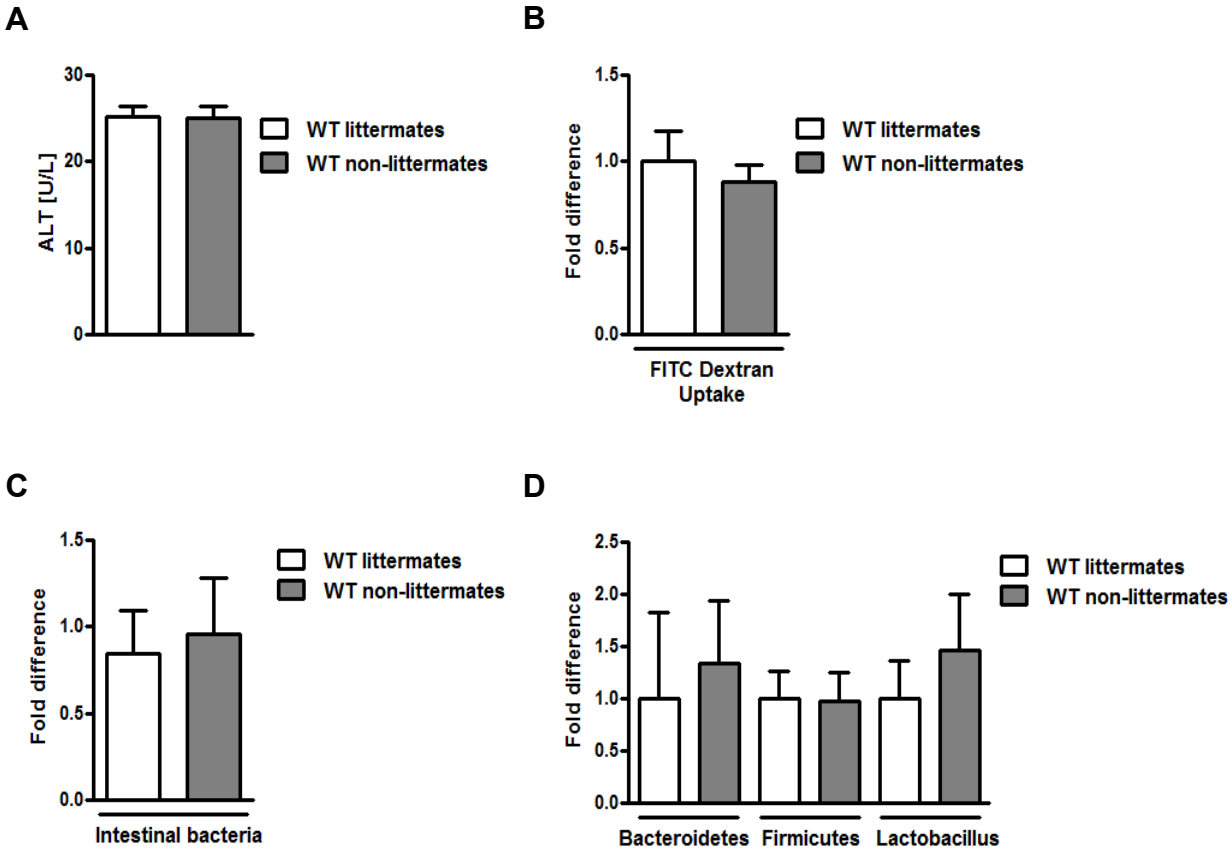
Supplementary Figure 1. Mucin-2 is abundantly expressed in the small and large intestine of wild type mice but undetectable in the liver. (A) Gene expression of Muc2 in several organs of wild type mice (n=3-5). (B) Immunofluorescent detection of Muc2 in jejunal sections of wild type and Muc2$^{-/-}$ mice, and in a wild type liver section.

Supplementary Figure 2



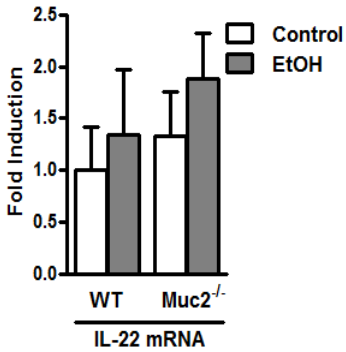
Supplementary Figure 2. Liver/body weight ratio and plasma triglycerides in wild type and Muc2$^{-/-}$ mice after feeding of an isocaloric diet or alcohol. Wild type or Muc2$^{-/-}$ mice were fed an intragastric isocaloric diet (n=2-6) or alcohol (n=7-10) for one week. (A) For the liver/body weight ratio, liver weight is expressed as a percentage of body weight. (B) Plasma triglycerides. *$p < 0.05$.

Supplementary Figure 3



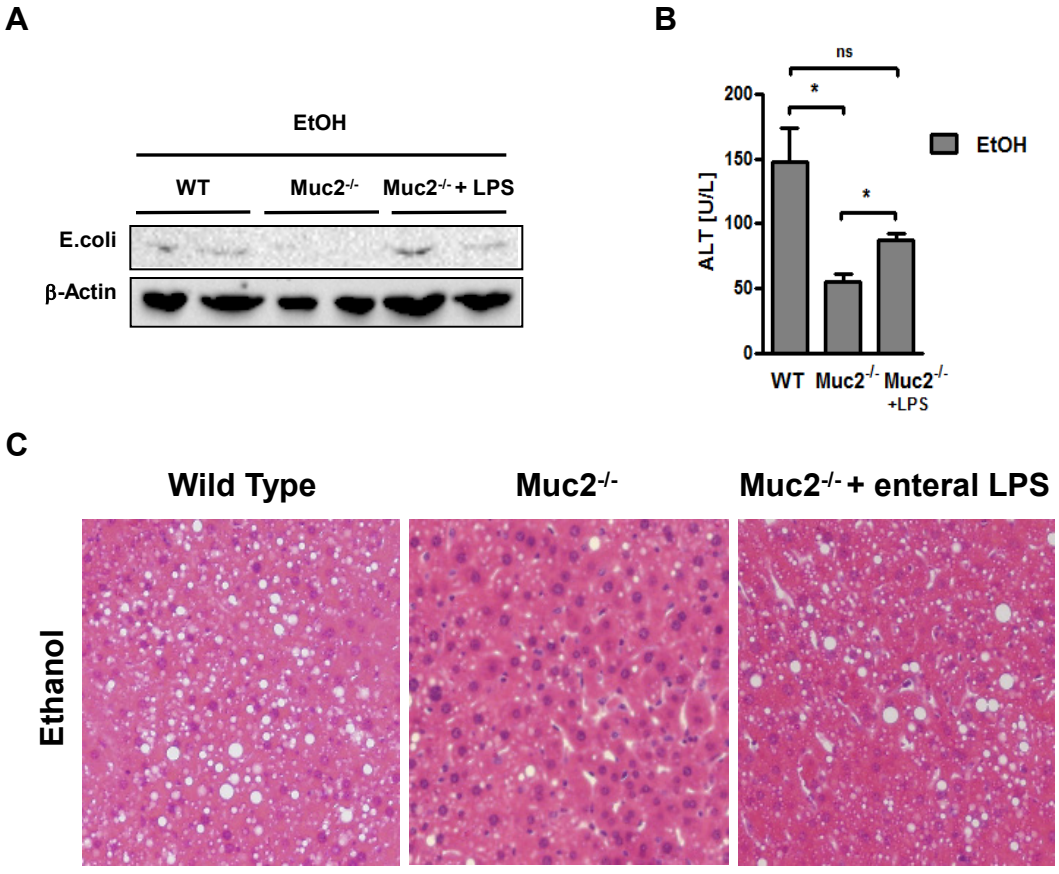
Supplementary Figure 3. Littermate and non-littermate wild type show a similar intestinal microflora profile. Littermate (n=3-4) and non-littermate (n=5) wild type C57BL/6J mice were compared. (A) Plasma ALT levels. (B) FITC-dextran in plasma 4 hrs after gavage. (C and D) Cecal levels of total bacteria, Bacteroidetes, Firmicutes and *Lactobacillus.*

Supplementary Figure 4



Supplementary Figure 4. Intestinal IL-22 gene expression. Wild type or Muc2$^{-/-}$ mice were fed an intragastric isocaloric diet (n=4-6) or alcohol (n=9-10) for one week. Jejunal gene expression of IL-22 was determined by QPCR.

Supplementary Figure 5



Supplementary Figure 5. Enteral LPS administration exacerbates experimental alcoholic steatohepatitis in Muc2$^{-/-}$ mice. Mice were fed alcohol alone, or alcohol and LPS via an intragastric feeding tube for one week (n=15 for wild type mice, n=8 for Muc2$^{-/-}$ mice and n=14 for Muc2$^{-/-}$ mice +LPS). (A) Western blot for *E.coli* protein in the liver. ß-Actin was used as a loading control. (B) Plasma ALT levels. (C) Photomicrographs of H&E stained livers. ns = not significant; *p<0.05.

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