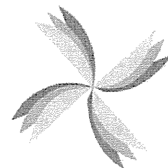
今月の話題



胆管癌と鑑別を要する良性胆管狭窄

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はじめに

胆道系上皮を被覆する胆管上皮は，腫瘍性のみならず炎症性疾患においても過形成や核濃染等の反応性変化を示し，また前癌病変である biliary intraepithelial neoplasia (BilIN) 病変もしばしば出現する．画像診断が進歩した昨今においても良悪の鑑別に苦慮する胆管狭窄症例は未だ少なくなく，術前の病理診断に委ねられる症例が多い．しかし，胆管生検では診断に十分な検体量が提出されることは少なく，また細胞診でも多彩な像を呈するため，我々病理医にとって少し気が重い領域である．今月の話題として，良性胆管狭窄をきたす疾患の最近の知見と胆管および肝生検診断時における注意点を交えて概説したい．

I．胆管癌として外科切除された良性胆管狭窄症例

胆道生検や細胞診で，腺癌疑いまたは偽陽性と診断し，胆管癌の診断で手術したにもかかわらず，手術材料では病理学的に明らかな癌を認めない症例に遭遇する．Fujita らの報告[1]によると176例の胆道手術症例のうち5例 (2.8%) が良性硬化性胆管炎であり，同論文の文献的集計でも5～17%となっており比較的頻度が高い．また，昨年度の日本胆道学会でもパネルディスカッションが企画され各施設の成績が発表されたが，本邦では胆道系手術症例の3%前後が術前の診断とは異なり良性胆管狭窄であったとの結果が出されている．術後の病理診断としては，原発性硬化性胆管炎 primary sclerosing cholangitis (PSC)，IgG4関連硬化性胆管 (IgG4-SC)，肝内結石症等の独立した疾患概念を有する疾患に加え，近年症例報告がされている濾胞性胆管炎 follicular cholangitis やいずれにもあてはまらない非特異的胆管炎の症例も多い．実際の診断時には，このような疾患に加えて，他疾患または病態に起因する二次性 (続発性) 硬化性胆管炎の鑑別も重要であり，胆道系手術の既往や感染症の可能性についても病理診断時には重要な臨床情報である．これら基本的な病態は，炎症細胞浸潤を伴いつつ胆管周囲の線維性硬化と胆管内腔の狭窄，閉塞であり，病態によっては胆管の進行性破壊と消失もみられ，最終的に胆汁性肝硬変をきたす．臨床データ上，IgG4-SC では血中 IgG4 高値を示すが，その他の硬化性胆管炎では病気や病態を反映する特異的なマーカーはない．

II．PSC

PSC の基本的病態は，肝門部大型胆管～肝外胆管レベルの硬化性胆管炎とそれに引き続いて起こる胆管閉塞である．しかし，このような胆管の変化は，胆管系外科手術や胆道結石に起因する二次性硬化性胆管炎でもみられ，潰瘍性大腸炎等の炎症性腸疾患に合併する症例以外では除外診断が重要である．欧米では PSC の62～100%に炎症性腸疾患を合併するとされている．本邦では34% (2012年)[2] と低率であるが，特に若年者では欧米の PSC に類似して潰瘍性大腸炎の合併率が高い．以前より本邦の PSC は若年と高齢者の2峰性の分布を示すことが知られていたが，近年，IgG4関連疾患の概要が明らかとなるにつれ，高齢者 PSC に IgG4-SC 症例の混入が懸念されていた．しかし，2012年の IgG4-SC を排除した PSC の全国調査でも，35～40歳と65～70歳代にピークを示す二峰性分布を示すことが明らかとなり[2]，若年群と高齢群における PSC の病態の異同が今後注目すべき課題である．PSC の診断には依然として除外診断が重要であり，臨床情報，胆道造影，病理組織学的所見を併せた総合的な診断が要求される．組織学的には胆管周囲の同心円状のタマネギ状線維化が特徴的であり，教科書で必ず提示される組織像である．しかし，末梢小型胆管にも類似の線維化が，IgG4-SC 等の肝内大型胆管～肝外胆管レベルの胆管障害に随伴して出現するため，肝針生検でみられるような小葉間胆管～隔壁胆管レベルの末梢小型胆管に出現する胆管周囲線維化は PSC と IgG4-SC の両者を鑑別する特徴的な所見ではない[3]．一方，胆道生検は悪性腫瘍の有無，IgG4-SC における IgG4 陽性細

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胞の有無についての情報を得ることができ，除外診断の一助となる．

Ⅲ．IgG4-SC

IgG4-SCは，全身性IgG4関連疾患の胆管病変としてとらえることができ，他臓器のIgG4関連疾患と同様に血中IgG4高値（通常135 mg/dL以上），高度のIgG4陽性形質細胞浸潤，ステロイド治療が著効といった共通の特徴を示し，それに加えて線維化による硬化性変化も臓器特異的な組織所見としてみられる．通常，単独で発症する症例は稀で，多くはIgG4関連の1型自己免疫性膵炎を合併する．高齢男性に好発するのが特徴であり，男女比は3：1である，平均年齢は69歳である[2]．病因については未だ不明であるが，好酸球増多や自己抗体の出現，免疫抑制剤の有効性より，病因として何らかの外来抗原または自己抗原に対するアレルギー反応や自己免疫機序が推定されている．基本的な病理所見として，IgG4陽性細胞浸潤のほかに，閉塞性静脈炎 obliterative phlebitis，花むしろ様線維化 storiform fibrosisが診断基準の所見としても挙げられている特徴的な所見である[4]．炎症はリンパ球形質細胞を主体とする慢性炎症細胞浸潤からなり，リンパ濾胞形成やしばしば好酸球浸潤が目立つ症例もある．また，PSCと異なり炎症の主座は粘膜面よりは胆管壁結合織にあり，胆管上皮には好中球浸潤を伴うびらん性変化は乏しく，胆管被覆上皮は剝離せずに比較的保たれていることが多い．このような所見は，胆道生検や擦過細胞診を鏡検する際の参考となる．また，PSC，胆管癌でも血中IgG4高値例や病変部でIgG4陽性細胞が目立つ症例があり，注意を要する．PSCは胆管癌の先行病変として重要であり，BilINの異型上皮もしばしばみられ，生検や細胞診で胆管癌疑いや偽陽性と診断される症例がある．また，IgG4関連疾患に胆管癌や前癌病変を伴う症例も稀であるが報告されている．さらに，血中IgG4低値のIgG4関連疾患や標的臓器でのIgG4陽性細胞浸潤が乏しいIgG4関連疾患なども報告されており，このような非定型例の取り扱いについては今後の課題である．

Ⅳ．二次性硬化性胆管炎

胆道結石，炎症性ポリープ，腫瘍，膵疾患，動脈瘤，胆道手術などが原因で閉塞機転による胆管狭窄をきたし，胆道閉塞が長期化すると，胆汁うっ滞や胆管炎により二次性（続発性）硬化性胆管炎へ進展する．反復性の細菌性胆管炎（上行性胆管炎）もしばしば合併する．組織学的に，大型胆管では慢性および化膿性炎症を伴いつつ胆管粘膜はびらんを呈し，残存する胆管上皮には種々の程度の過形成やBilIN病変などを認める．近年，IgG4-SCとの類似性が指摘されている濾胞性胆管炎は，肝門部胆管を中心とする病変で，胆管の狭窄は限局性に生じ，胆管周囲における著明な芽中心を伴うリンパ濾胞形成と密な線維化が特徴である[5]．コンサルト症例を含めた自験例では多少なりともIgG4陽性細胞を散見し，IgG4-SCとの鑑別を要する症例もあり，また文献的に免疫抑制剤が奏効する症例もある．

おわりに

胆管生検や細胞診，また胆管断端の術中迅速診断は我々病理医にとって荷が重い検体であり，画像所見や臨床診断に流されやすい局面もある．明らかな癌があれば明確な診断が可能であるが，それ以外は異型上皮や偽陽性などのグレーゾーンが多い領域であり，実際に手術検体をみてもグレーゾーンの症例が多い．逆に生検や細胞診で明らかな癌がでなくても，手術検体は癌であった症例も多数存在する．臆することなく診断し，日頃からの臨床医との意思疎通が重要である．

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医療 up-to-date

良性胆管狭窄を来す胆道系炎症性疾患

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はじめに

本稿を御拝読していただく石川県医師会の先生方に、まず自己紹介をさせて頂きたいと存じます。私は、平成26年５月１日付で、中沼安二前教授が主宰されていた金沢大学医薬保健研究域医学系形態機能病理講座（病理学第二）の教授を拝命いたしました。私は平成３年に金沢大学医学部を卒業し、中沼先生の門下生として肝臓病理学の診断・研究に一貫して従事してまいりました。今後も教室の伝統を継承しつつ、肝臓の組織形態学に加え、近年のいわゆるオミクス解析および遺伝子診断にも対応しつつ、肝臓学の発展に寄与するよう尽力して参ります。

さて、本稿では私どもの専門分野のひとつである胆道系疾患、特に胆管癌との鑑別が重要な胆管炎疾患についてご紹介致します。胆道系上皮を被覆する胆管上皮は、腫瘍性のみならず炎症性疾患においても増生や核濃染等の変化に富む所見を示し、胆道生検や胆汁細胞診にて良悪判定困難な検体が多く見られます。また、最新の WHO 分類（2010年）では、胆管癌の前癌病変である胆管上皮内腫瘍（biliary intraepithelial neoplasia, BilIN）や前浸潤癌病変である胆管内乳頭状腫瘍（intraductal papillary neoplasm of bile duct, IPNB）も疾患名として採用され、ますます鑑別すべき疾患・病態が増えてきております。画像診断が進歩した昨今においても良悪の鑑別に苦慮する胆管狭窄症例は未だ少なくなく、術前の病理診断でも良悪判定困難な症例に遭遇します。近年、胆道系炎症性疾患の症例数の蓄積と認知の普及により、良性胆管狭窄を来たす疾患群の概要が明らかとなり、胆道系腫瘍と鑑別すべき疾患の臨床病理学的特徴について最近の知見を御紹介します。

良性胆管狭窄の概要

胆管炎という病態は、腫瘍等による閉塞機転、塞栓術、動注化学療法、胆道系手術等による胆管炎、また胆道感染症などの原因が明らかな胆管炎に加えて、原発性硬化性胆管炎、IgG４関連硬化性胆管炎、また小児の胆道閉鎖症、胆道拡張症の独立した疾患概念を有する原因不明な炎症性胆道系疾患もあり、様々な原因・病態から発生します。特に胆管癌との鑑別が重要でありますが、胆管癌であっても胆道生検や細胞診で明らかな腺癌組織や腺癌細胞が確認出来る症例はむしろ少なく、逆に腺癌疑いまたは疑陽性と診断し、胆管癌の疑いで手術したにもかかわらず手術材料では病理学的に明らかな癌を認めない症例にも遭遇します。文献的に、胆道手術症例のうち５～17％が良性の胆管炎であり、2013年の胆道学会における本邦のデータでも胆道系手術症例の３％前後が術前の診断とは異なり良性胆管狭窄であったとの報告がなされています。

良性胆管狭窄を来す疾患

IgG 4 関連硬化性胆管炎：IgG 4 関連疾患は、2001年信州大学の浜野先生らによって発信された疾患で、近年 IgG 4 関連疾患の認知が広まるにつれて、疾患の蓄積とともに病態も明らかとなってきました。2007年 IgG 4 研究会が発足し、金沢大学リウマチ膠原病内科の川野先生が代表世話人をされています。IgG 4 関連疾患は全身性の疾患で、自己免疫性膵炎、硬化性胆管炎、後腹膜線維症、炎症性偽腫瘍、キュッナー腫瘍として古くから知られていた疾患が IgG 4 関連疾患のカテゴリーに含まれることがわかってきました。臨床的には、血中 IgG 4 値の高値（通常135mg/dl 以上）、高度の IgG 4 陽性形質細胞浸潤、ステロイド治療が著効といった特徴を示し、それに加えて線維化による硬化性変化も臓器特異的な組織所見として見られます。胆道系においても IgG 4 関連疾患が存在し、IgG 4 関連硬化性胆管炎の呼称で現在分類されています。IgG 4 関連硬化性胆管炎の多くは、IgG 4 関連の 1 型自己免疫性膵炎に合併して見られます。臨床的には、高齢男性に好発し、胆管狭窄に関連した臨床症状を呈するのが特徴です。IgG 4 関連疾患の病因については未だ不明ですが、好酸球増多や自己抗体の出現、免疫抑制剤の有効性より、なんらかの外来抗原または自己抗原に対するアレルギー反応や自己免疫機序が病因として推定されています。病期の進展とともに胆管壁の硬化性肥厚と胆管狭窄が見られ、臨床的には下記の原発性硬化性胆管炎や胆道癌に類似した臨床像および画像所見を示しますが、ステロイドが著効することが本疾患の特徴であり、常に IgG 4 関連疾患を鑑別に挙げつつ正確な鑑別診断をすることが非常に重要であります。

原発性硬化性胆管炎（PSC）：医師国家試験でも出てくるよく知られた疾患名ですが、稀な疾患です。組織学的に胆管周囲の同心円状のタマネギ状線維化が特徴的であり、教科書で必ず提示される組織像です。欧米では PSC の62～100％に炎症性腸疾患を合併するとされています。本邦では34％（2012年データ）と低率ですが、特に若年者では欧米の PSC に類似して潰瘍性大腸炎の合併率が高いのが特徴です。また、以前より本邦の PSC は若年と高齢者の 2 峰性の分布を示すことが知られておりましたが、IgG 4 関連疾患の概要が明らかとなるにつれ、高齢者 PSC に IgG 4 関連硬化性胆管炎症例の混入が懸念されていました。しかし、2012年の IgG 4 関連硬化性胆管炎を排除した PSC の全国調査でも、35－40歳と65－70歳台にピークを示す二峰性分布を示すことが明らかとなり、若年群と高齢群における PSC の病態の異同が今後注目すべき課題であります。いずれにせよ PSC の基本的病態は、肝門部大型胆管～肝外胆管レベルの硬化性胆管炎とそれに引き続いて起こる胆管閉塞です。しかし、このような胆管の変化は、胆管系外科手術や胆道結石に起因する 2 次性硬化性胆管炎でも見られ、潰瘍性大腸炎等の炎症性腸疾患に合併する症例以外は除外診断が重要であり、臨床情報、胆道造影、病理組織学的所見を併せた総合的な診断が要求されます。また、PSC は胆管癌の先行病変としても重要であり、異型上皮である胆管上皮内腫瘍（BilIN）もしばしば見られ、生検や細胞診で胆管癌疑いや疑陽性と診断される症例があります。

二次性胆管炎：胆道結石、炎症性ポリープ、腫瘍、膵疾患、動脈瘤、胆道手術などが原因で胆管狭窄を来たし、胆道閉塞が長期化すると胆汁うっ滞や胆管炎により二次性（続発性）胆管炎へと進展し、硬化性変化も加わると PSC に類似した病態を呈します。

成因不明であるが特徴的な慢性胆管炎：病態発生になんらかの免疫学的機序が推測される原因不明の硬化性胆管炎として、肥満細胞性胆管炎（Mast cell cholangitis）、好酸球性胆管炎（Eosinophilic cholangitis）、濾胞性胆管炎（Follicular cholangitis）と呼称される特徴的な組織像を呈する胆管炎がありますが、いずれも症例報告レベルの稀な疾患です。

非特異的な慢性胆管炎：臨床的に閉塞性黄疸を来たし、PSC や IgG 4 硬化性胆管炎、 2 次性胆管炎等の特徴的所見はなく、胆汁細胞診や胆道生検で確定診断が得られないことから悪性腫瘍を否定しきれず手術となる症

例は稀ではありません。しかし、切除検体には、胆道狭窄部に非特異的な慢性胆管炎と反応性の上皮過形成しか存在しない症例や狭窄・閉塞そのものが確認出来ない症例も存在します。

最後に

胆管生検や細胞診、また胆管断端の術中迅速診断は我々病理医にとって荷が重い検体あり、画像所見や臨床診断に流され易い局面もあるのが実状です。明らかな癌があれば明確な診断が可能ですが、それ以外は異型上皮や疑陽性などのグレーゾーンが多い領域であり、実際に手術検体を見ても境界病変の症例もあります。逆に生検や細胞診で明らかな癌がでなくても、手術検体は癌であった症例も経験します。是非、胆道系の病理検体を提出される際には、原発性硬化性胆管炎や IgG 4 関連硬化性胆管炎の可能性のみならず、二次性胆管炎の鑑別のために胆道系手術の既往や感染症の可能性についても重要な臨床情報としてお知らせ頂きますよう御願い申し上げます。

Infection and Immunity

Blockade of Indoleamine 2,3-Dioxygenase Reduces Mortality from Peritonitis and Sepsis in Mice by Regulating Functions of CD11b $^+$ Peritoneal Cells

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Blockade of Indoleamine 2,3-Dioxygenase Reduces Mortality from Peritonitis and Sepsis in Mice by Regulating Functions of CD11b$^+$ Peritoneal Cells

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Indoleamine 2,3-dioxygenase-1 (Ido), which catalyzes the first and limiting step of tryptophan catabolism, has been implicated in immune tolerance. However, the roles of Ido in systemic bacterial infection are complicated and remain controversial. To explore this issue, we examined the roles of Ido in bacterial peritonitis and sepsis after cecal ligation and puncture (CLP) in mice by using the Ido inhibitor 1-methyl-D,L-tryptophan (1-MT), by comparing Ido$^{+/+}$ and Ido$^{-/-}$ mice, or by using chimeric mice in which Ido in the bone marrow-derived cells was deficient. Ido expression in the peritoneal CD11b$^+$ cells and its metabolite L-kynurenine in the serum were increased after CLP. 1-MT treatment or Ido deficiency, especially in bone marrow-derived cells, reduced mortality after CLP. Compared to Ido$^{+/+}$ mice, Ido$^{-/-}$ mice showed increased recruitment of neutrophils and mononuclear cells into the peritoneal cavity and a decreased bacterial count in the blood accompanied by increased CXCL-2 and CXCL-1 mRNA in the peritoneal cells. Ido has an inhibitory effect on LPS-induced CXCL-2 and CXCL-1 production in cultured peritoneal cells. These findings indicate that inhibition of Ido reduces mortality from peritonitis and sepsis after CLP via recruitment of neutrophils and mononuclear cells by chemokine production in peritoneal CD11b$^+$ cells. Thus, blockade of Ido plays a beneficial role in host protection during bacterial peritonitis and sepsis.

Sepsis is a systemic inflammatory response syndrome induced by microbial infection (1, 2). The pathogenesis of sepsis involves a progressive and dynamic expansion of a systemic inflammatory response to bacterial infection (3). Neutrophils are major leukocytes that are promptly recruited to the inflamed site in response to infection or tissue injury. These cells are ideally suited to the elimination of pathogenic bacteria owing to their capability for phagocytosis and releasing the stores of granular lytic enzymes and antimicrobial polypeptides into the phagolysosome (4). In this way, migrating neutrophils may control bacterial growth and, consequently, prevent bacterial dissemination and death of the host (5). However, once the host fails to restrict the pathogens to a localized area, the pathogens and/or their products may spread systemically and result in the death of their host (6). Previous reports have demonstrated that the severity of sepsis induced by cecal ligation and puncture (CLP) (7) or by *Staphylococcus aureus* inoculation (8) is closely associated with reduced neutrophil migration to the infection focus. Therefore, to inhibit bacterial growth in the local site, neutrophils must migrate to the site of infection in response to chemotactic factors, such as chemokine (C-X-C motif) ligand 2 (CXCL-2) and chemokine (C-X-C motif) ligand 1 (CXCL-1). These chemokines, which are secreted from macrophages, neutrophils, and epithelial cells in response to endotoxin and various proinflammatory cytokines (9, 10), have been identified as chemoattractants of neutrophils *in vitro* and *in vivo* (11, 12) and have pathophysiological roles in several inflammatory disease states, including endotoxemia-induced lung injury (13), glomerulonephritis (14), and bacterial meningitis (15).

Indoleamine 2,3-dioxygenase-1 (Ido), which catalyzes the first and limiting step of tryptophan (Trp) catabolism, is induced in various cell types during infection, especially in response to gamma interferon (IFN-γ) signaling and/or bacterial components, such as Toll-like receptor (TLR) ligands, and plays a pivotal role in immune tolerance (16). Ido has been thought to have beneficial effects. For example, recent studies have shown that the TLR3 ligand poly(I·C) induces Ido activation in astrocytes, resulting in an antiviral response (17). IFN-γ-induced Ido also has an antiviral effect in measles virus infection of epithelial and endothelial cells (18). However, disadvantageous functions of Ido have also been reported. Ido is expressed in human (19) and mouse (20) tumor cells, dendritic cells (DCs) (16), and macrophages following microbial (21) or viral (22) infections, and Ido inhibition improves the pathophysiology of those ailments. Favorable effects of Ido inhibition are also reported in human immunodeficiency virus (HIV)-infected patients (23) and in major-trauma patients (24). Ido activation by CTLA-4 stimulation from the regulatory T cells or DCs is involved in viral increase in tissues from simian immunodeficiency virus (SIV$_{mac251}$)-infected macaques (25). The Ido inhibitor 1-methyl-D,L-tryptophan (1-MT) may enhance anti-HIV immunity (26). Ido blockade protects mice against lipopolysaccharide (LPS)-induced endotoxin shock, in association

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with modulation of interleukin 12 (IL-12) and IL-10 production in DCs (27). In addition, several other studies suggest that Ido-expressing cells deplete Trp from the extracellular milieu and secrete Trp metabolites (including L-kynurenine [L-Kyn], 3-hydroxy-kynurenine, 3-hydroxyanthranilic acid, and quinolonic acid), which induce T cell apoptosis and suppress immune responses *in vitro* (28–30). Therefore, whether induction of Ido always has beneficial effects for the host and how Ido induces immune tolerance are not clear.

The CLP model is the most widely used rodent model for experimental peritonitis and sepsis. The model is considered to be realistic for the induction of polymicrobial sepsis in experimental settings to study the underlying mechanisms of sepsis (31). In this study, the roles of Ido in immune regulation were examined in polymicrobial sepsis induced by CLP by using $Ido^{-/-}$ mice or the Ido inhibitor 1-MT. We demonstrated that the induction of CXCL-1 and CXCL-2 in peritoneal cells by CLP was increased by Ido inhibition, resulting in increased recruitment of neutrophils and mononuclear cells to the local infectious focus, which suppresses progression to sepsis.

MATERIALS AND METHODS

Mice. Eight- to 10-week-old male mice were used in this study. Ido1 gene-deficient ($Ido^{-/-}$) mice on a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice that were homozygous null ($Ido^{-/-}$) by targeted disruption of the Ido gene were selected from the offspring of heterozygous-homozygous matings by genotyping by performing PCR of tail DNA. C57BL/6J mice obtained from Japan SLC (Shizuoka, Japan) were used as wild-type ($Ido^{+/+}$) controls. For blockade of Ido, mice were administered 1-MT (5 mg/ml) in their drinking water starting from 3 days before or from just after CLP, and on average, each mouse consumed 3.5 ml/day. L-Kyn was administered as described previously (19, 32). Briefly, mice were intraperitoneally injected with L-Kyn (20 mg/kg of body weight; Sigma-Aldrich) 24 h before CLP. All animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of Gifu University (approval number 26-8).

Mouse sepsis model. Sepsis was induced by CLP as previously reported (33). Briefly, mice were anesthetized by intraperitoneal administration of pentobarbital diluted in PBS (1.25 mg/ml; 200 μl), and a midline incision (1 cm) was made on the anterior abdomen. The cecum was exposed and ligated below the ileocecal junction without causing bowel obstruction. A single puncture was made using a 22-gauge needle to induce septic injury. Pressure was applied (the cecum was squeezed) to allow the cecum contents to be expressed through the puncture. The cecum was placed back in the abdominal cavity, and the peritoneal wall and skin incision were closed. The sham-operated animals underwent identical laparotomy without cecum ligation or puncture. Mortality after CLP was monitored in $Ido^{-/-}$ mice ($n = 25$), $Ido^{+/+}$ mice ($n = 23$), 1-MT-treated mice ($n = 17$), and vehicle-treated mice ($n = 15$). To obtain samples, the animals were anesthetized and humanely killed at the indicated times. In another experimental animal model, sepsis was induced by inoculation of *S. aureus* (ATCC 25923). Briefly, mice were intravenously injected with *S. aureus* (1×10^8 CFU in 100 μl phosphate-buffered saline [PBS]). Mortality after the infection was monitored in $Ido^{-/-}$ mice ($n = 12$), $Ido^{+/+}$ mice ($n = 12$), 1-MT-treated mice ($n = 12$), and vehicle-treated mice ($n = 12$).

Bacterial counts in the peritoneal exudate and in blood. The peritoneal exudate (which was harvested by introducing 1.5 ml of PBS-EDTA [1 mM]), blood, and feces were collected under sterile conditions, and aliquots of serial dilutions of these samples were planted on brain heart infusion agar plates (Biovalley, Marne la Vallée, France). The plates were incubated at 37°C in a 5% CO_2 atmosphere, and CFU were counted after 18 h. The results are expressed as the median log CFU per ml of the peritoneal exudate or blood and as the median log CFU per mg of feces. The samples were collected from sham-operated ($n = 6$) or CLP-operated ($n = 7$) $Ido^{-/-}$ or $Ido^{+/+}$ mice.

Bacterial killing by neutrophils. The bactericidal capacity of neutrophils was assessed as previously reported (34). Briefly, $Ido^{+/+}$ and $Ido^{-/-}$ mice were intraperitoneally injected with thioglycolate (4%) to obtain peritoneal neutrophils. The induced neutrophils were harvested 2 h later by washing the peritoneal cavities with PBS. Cell viability was >98%, and the cell population consisted of neutrophils, representing >85% of the total leukocytes.

The bacterial suspension (*Escherichia coli* ATCC 25922) was added to the isolated $Ido^{+/+}$ and $Ido^{-/-}$ neutrophil culture (2×10^6 bacteria and 1×10^6 neutrophils in 1 ml PBS) in 1.5-ml tubes, and the mixture was incubated for 3 h at 37°C with mild shaking. As a control, the bacterial suspension was incubated under the same experimental conditions in the absence of neutrophils. Bacterial viability was assessed by serial log dilutions and plating on brain heart infusion agar plates. CFU were counted after 18 h, and the results are expressed as the number of viable bacteria.

Leukogram. Blood diluted in EDTA and peritoneal exudate were collected 6 h after CLP. Total counts were performed using a Neubauer chamber. Differential cell counts were conducted on slides stained with May-Gruenwald-Giemsa (Sysmex, Kobe, Japan). Differential counts were performed under oil immersion microscopy, where 200 cells were counted for the determination of the percentage of neutrophils and mononuclear cells present in blood and peritoneal exudate. The results are expressed as means and standard deviations (SD) of cells counted per milliliter.

Measurements of L-Kyn. L-Kyn was measured using high-performance liquid chromatography (HPLC) with a spectrophotometric detector (UV-8000; Tosoh, Tokyo, Japan) as described previously (19, 32).

Cell preparation and culture. Cells from the peritoneal cavity were harvested by introducing 1.5 ml of PBS-EDTA (1 mM). The isolated peritoneal cells were plated on 96-well culture plates (1×10^5 cells/well) or sterile slides in RPMI 1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Boehringer Mannheim Biochemica, Manheim, Germany), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO_2 for 2 h and washed with PBS. In some experiments, fractionation of the isolated peritoneal cells was performed using MACS MagneticBead columms (Miltenyi Biotec, Bergisch Gladbach, Germany) with antibodies against CD11b, CD11c, Gr-1, and DX5, according to the manufacturer's instructions. For LPS treatment, the cells were then treated with 0.1 μg/ml LPS from *E. coli* O55:B5 (Sigma-Aldrich, St. Louis, MO, USA) for 6 h.

Quantitative real-time reverse transcription (RT)-PCR. Total RNA was isolated from peritoneal cells from sham- or CLP-operated mice, from cultured peritoneal cells treated or not with LPS for 6 h, or from fractionated cells using the RNeasy minikit (Qiagen, Hilden, Germany).

The isolated RNA was transcribed into cDNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was used as a template for real-time RT-PCR, along with primer-probe sets for Ido, CXCL-2, CXCL-1, MCP-1, CXCL-5, and IL-17 (TaqMan Gene Expression Assays; Applied Biosystems) and 2× TaqMan Universal PCR master mix (Applied Biosystems) according to the manufacturers' recommendations. The expression levels of the respective genes were normalized to those of 18S rRNA (Applied Biosystems) as an internal control in the reaction. All reactions were performed in duplicate. The sample value was expressed as the median interquartile range (IQR) and shown as a scatter plot.

Determination of chemokine levels. CXCL-2 and CXCL-1 levels were detected in the sera of mice that were operated on or in the culture media for peritoneal cells by enzyme-linked immunosorbent assay (ELISA) according to the recommendations of the manufacturer (R&D Systems, Minneapolis, MN, USA).

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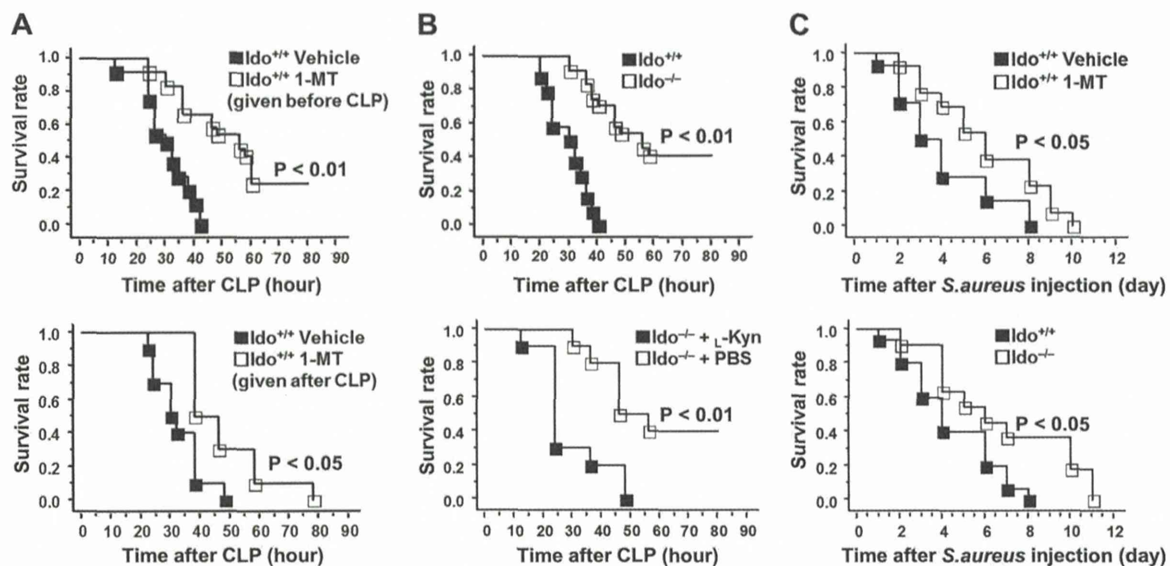

FIG 1 Inhibition or deficiency of Ido suppresses mortality after sepsis. (A) Wild-type mice underwent CLP. 1-MT or vehicle was administered 3 days before (top) or a few hours after (bottom) surgery. (B) $Ido^{+/+}$ or $Ido^{-/-}$ mice underwent CLP (top). L-Kyn was administered 24 h before CLP in $Ido^{-/-}$ mice (bottom). *S. aureus* was inoculated into $Ido^{+/+}$ or $Ido^{-/-}$ mice treated or not with 1-MT. The survival rates of the animals are shown. Statistically significant differences between the groups were determined using the log-rank test.

Immunofluorescence assay. Cells on the slides were fixed in 4% paraformaldehyde for 20 min. After incubation with 0.1% Triton X-100 for 30 min, the cells were blocked using 1% bovine serum albumin (BSA) and 0.1% Tween 20 containing PBS. Subsequently, the cells were incubated with primary antibodies against Ido and CD11b (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 4°C. The anti-Ido polyclonal antibody was generated by the peptide H-CMKPSKKKPTDGDKS-OH in rabbits as described previously (19, 32). After washing with 0.1% Tween 20 containing PBS, the cells were incubated with the secondary antibodies fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG (DakoCytomation, Carpinteria, CA, USA). 4,6-Diamino-2-phenylindole (Dojindo, Tokyo, Japan) was used for nuclear staining. The fluorescence was visualized using a BX51 fluorescence microscope equipped with a DP70 digital camera (Olympus, Tokyo, Japan). To determine the number of $CD11b^+$ cells in the peritoneal cavity, the percentage of $CD11b^+$ cells present in the peritoneal exudate was determined by counting 200 cells, and the total number of cells multiplied by the percentage of $CD11b^+$ cells was calculated as the total number of $CD11b^+$ cells.

BMT. Bone marrow (BM) transplantation (BMT) was performed on 5-week-old male mice as described previously (32). The recipient mice ($Ido^{+/+}$ and $Ido^{-/-}$ mice) were irradiated in fractionated doses (5 Gy twice with a 4-hour interval) and reconstituted with whole BM cells by injection (5×10^6 BM cells from $Ido^{+/+}$ or $Ido^{-/-}$ donor mice) via the tail vein. These BMT mice were maintained under specific-pathogen-free conditions and given 500 U/ml of gentamicin sulfate (Invitrogen, Carlsbad, CA, USA) and 100 μg/ml of polymyxin B sulfate (Kayaku Co., Tokyo, Japan) in drinking water for 4 weeks after BMT. The animals were subjected to CLP, and mortality was recorded (at least 20 mice died in each group).

Statistical analyses. The survival rates of mice were analyzed by the Kaplan-Meier method. Statistically significant differences between 2 groups were determined using Student's *t* test, and those among more than 3 groups were determined using one-way analysis of variance (ANOVA). Bacterial counts were reported as the median log CFU and were analyzed using the Mann-Whitney *U* test. StatView 4.5 software was used for these statistical analyses. The criterion for statistical significance was a P value of <0.05.

RESULTS

Ido inhibition suppressed CLP-induced mortality and reduced bacterial levels in the blood. We first examined whether inhibition of Ido has beneficial effects on reducing the impact of sepsis. Treatment with 1-MT, an inhibitor of Ido, given either before (Fig. 1A, top) or after (Fig. 1A, bottom) CLP improved the survival rate following surgery, as did the knockout of Ido (Fig. 1B, top). Administration of L-Kyn reversed the $Ido^{-/-}$ phenotype (Fig. 1B, bottom). Similarly, the inhibition or knockout of Ido also improved the survival rate of *S. aureus*-induced sepsis (Fig. 1C). Serum L-Kyn levels significantly increased after CLP in $Ido^{+/+}$ mice, and knockout or inhibition of Ido reduced the induction of L-Kyn (Fig. 2). These results indicate that L-Kyn production following Ido activation by CLP is involved in mortality. The bacte-

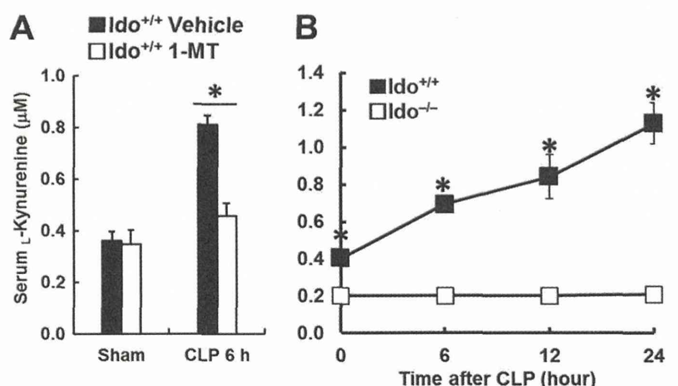

FIG 2 Inhibition or absence of Ido decreased the induction of serum L-Kyn levels after CLP. 1-MT-treated (24 h before CLP) and vehicle-treated (Sham) mice (A) or $Ido^{+/+}$ or $Ido^{-/-}$ mice (B) underwent CLP. The animals were humanely killed at the indicated times. Serum L-Kyn levels were measured by using HPLC. The data are means and SD from at least 5 independent experiments. *, $P < 0.01$ using ANOVA.

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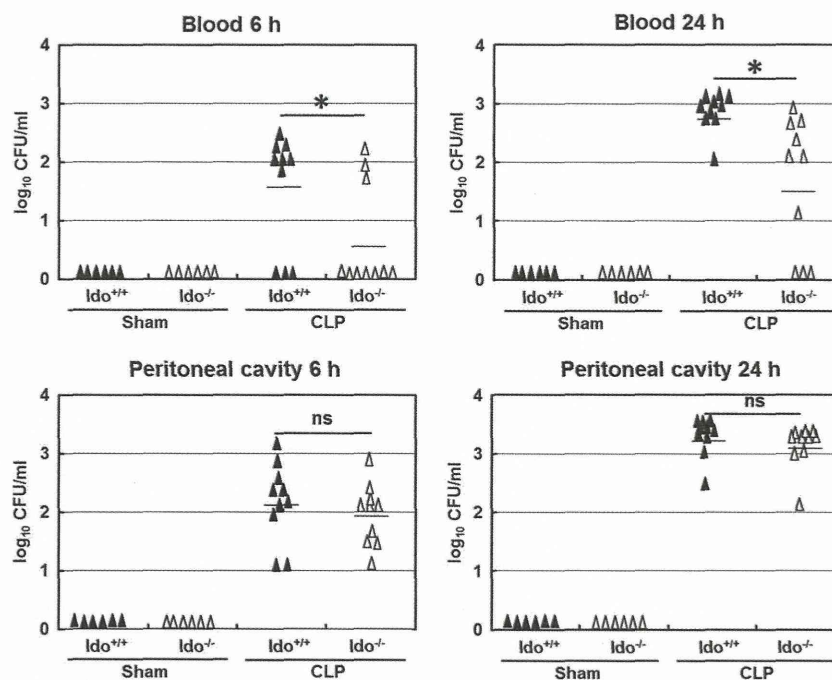

FIG 3 Ido is involved in the bacterial load after CLP. $Ido^{+/+}$ and $Ido^{-/-}$ mice underwent CLP. The numbers of bacteria in the blood (top) and in peritoneal exudate (bottom) were determined 6 or 24 h after CLP. The results are expressed as the median log CFU/ml in the peritoneal exudate or blood. *, $P < 0.05$ compared with $Ido^{+/+}$ CLP mice, using a Mann-Whitney *U* test. ns, not significant.

rial load in the peritoneal exudate from $Ido^{-/-}$ mice 6 or 24 h after CLP was comparable to that from $Ido^{+/+}$ mice (Fig. 3, bottom). In contrast, $Ido^{-/-}$ mice showed a marked decrease in the amount of bacteria in the blood compared with $Ido^{+/+}$ mice (Fig. 3, top). Furthermore, there was no difference in the bacterial load in feces between $Ido^{+/+}$ mice and $Ido^{-/-}$ mice (data not shown). In addition, Trp supplementation did not affect the intestinal bacterial growth on the minimal-medium plates (data not shown). These results suggest that CLP induces peritonitis and sepsis in $Ido^{+/+}$ mice, as previously reported (35–37), and that the bacterial infection is regulated in the local infection focus in $Ido^{-/-}$ mice, resulting in the improved survival rate.

To elucidate the mechanisms by which Ido contributes to the onset of sepsis, we assessed the numbers of blood and peritoneal-cavity circulating neutrophils and mononuclear cells 6 and 24 h after CLP. Although the increased numbers of blood circulating neutrophils and mononuclear cells were similar in $Ido^{+/+}$ and $Ido^{-/-}$ mice, those of peritoneal-cavity circulating neutrophils and mononuclear cells were significantly higher in $Ido^{-/-}$ mice than in $Ido^{+/+}$ mice (Table 1). Moreover, bacterial killing by the

TABLE 1 Numbers of circulating neutrophils and mononuclear cells in $Ido^{+/+}$ and $Ido^{-/-}$ mice after CLP

		No. of circulating cells[a]			
		$Ido^{+/+}$ mice		$Ido^{-/-}$ mice	
Location and cell type	Time (h)	Sham operated	CLP operated	Sham operated	CLP operated
Peritoneal cavity					
Neutrophils	6	0.024 ± 0.03	5.628 ± 4.96[b]	0.109 ± 0.09	18.758 ± 4.97[b,c]
	24	0.109 ± 0.05	6.263 ± 2.19[b]	0.171 ± 0.07	17.633 ± 3.34[b,c]
Mononuclear cells	6	0.607 ± 0.27	2.732 ± 0.72[b]	0.567 ± 0.74	8.261 ± 2.56[b,c]
	24	1.315 ± 0.09	2.314 ± 0.65[b]	1.159 ± 0.14	7.866 ± 2.99[b,c]
Blood					
Neutrophils	6	0.125 ± 0.04	1.479 ± 0.95[b]	0.075 ± 0.04	3.162 ± 1.47[b,c]
	24	0.156 ± 0.05	2.196 ± 0.74[b]	0.130 ± 0.03	3.829 ± 0.98[b,c]
Mononuclear cells	6	4.925 ± 1.20	2.584 ± 2.46	3.137 ± 1.18	4.144 ± 1.88[b,c]
	24	4.800 ± 0.87	2.073 ± 1.62	3.258 ± 0.86	3.652 ± 1.56[b,c]

[a] $Ido^{+/+}$ and $Ido^{-/-}$ mice were subjected to sham or CLP surgery. Peritoneal exudate and blood samples were collected at 6 or 24 h after the surgery, and the neutrophils and mononuclear cells in the peritoneal cavity (10^6/cavity) and blood (10^6/ml) were counted. The data are expressed as means ± SD from 10 independent experiments.
[b] $P < 0.05$ compared with sham-operated mice.
[c] $P < 0.05$ compared with CLP-operated $Ido^{+/+}$ mice using ANOVA.

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