報告された[4]。糖尿病での酸化ストレスの原因となる活性酸素種コントロールの重要性に注目が集まっている。

4) Nishikawa T, et al : Nature 404 : 787-790, 2000.

非酵素的糖化後期生成物

長く続く高血糖状態により，さまざまな蛋白が糖化される。この結果，AGEが生成される。AGEは，表1に示すような動脈硬化進展機序により，血管障害を引き起こす[5]。

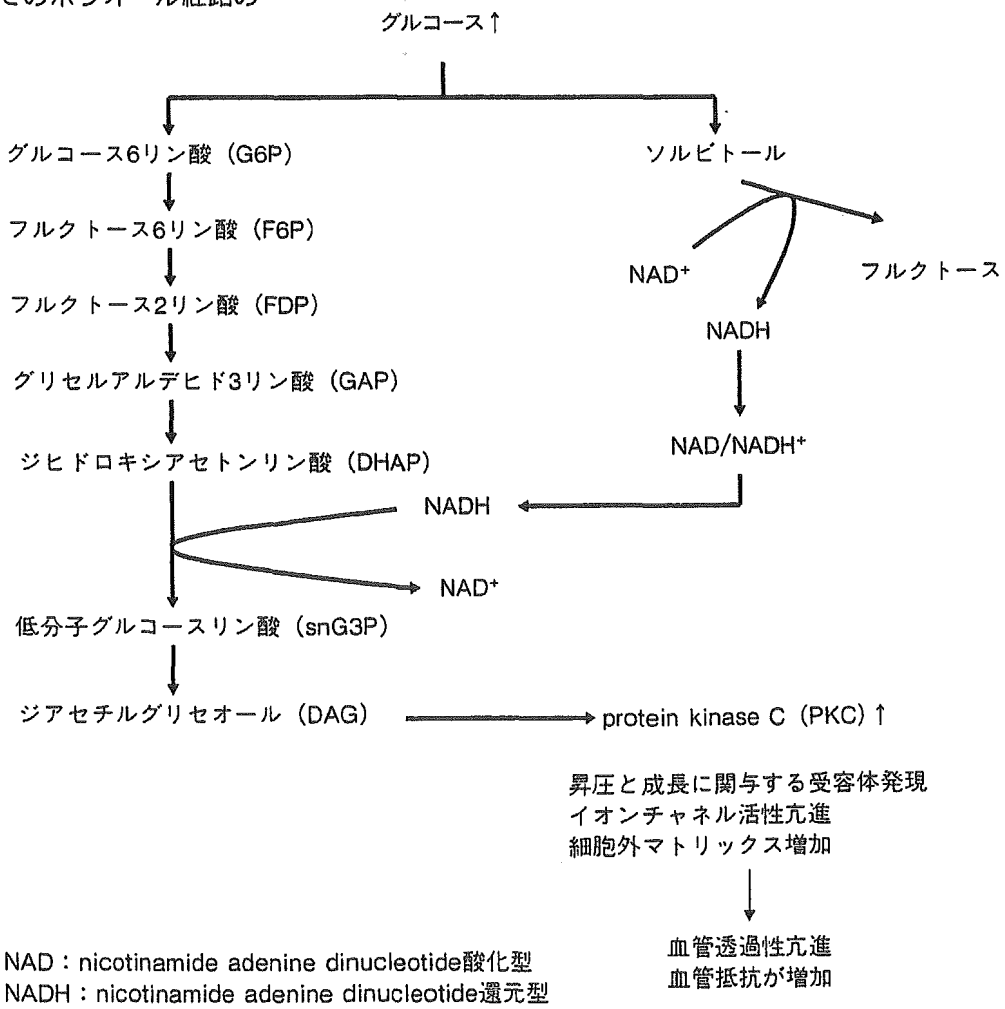
5) Farkouh ME, et al : The Heart (10th ed). McGraw - hill, 2001, p2197-2218.

血管内皮細胞の障害

高血糖，酸化ストレス，AGEなどにより血管内皮細胞が障害を受け，一酸化窒素による内皮依存性の血管拡張反応が低下する。インスリンは血管拡張を起こすことが知られている。一方，インスリン抵抗性はインスリンによる血管拡張反応を減弱させるため，血管トーヌスが亢進する。最近，血管内皮細胞が高血糖に曝露されて活性化するペルオキダーゼADPリボースポリメラーゼ（PARP）阻害により，内皮依存性血管拡張反応が改善することが報告されて[6]，PARPが，血管内皮細胞障害改善の鍵を握るものとして注目されている。

6) Soriano GF, et al : Nat Med 7 : 108 - 113, 2001.

図1　ブドウ糖過剰状態でのポリオール経路の活性化とPKC亢進



NAD：nicotinamide adenine dinucleotide酸化型
NADH：nicotinamide adenine dinucleotide還元型

血管平滑筋細胞と泡沫細胞形成

最近の分子生物学的検索によると，血管平滑筋細胞を高血糖に曝すと，MAP (mitogen-activated protein) キナーゼに属する酵素が活性化されることが明らかにされている[7]。この酵素は，細胞増殖やアポトーシスに関与して，動脈硬化進展をきたすと考えられている。糖尿病例で経皮的冠動脈形成術 (percutaneous coronary intervention：PCI) 後の再狭窄率が高い原因として，血管平滑筋細胞の異常反応が指摘されている。高血糖下での泡沫細胞の形成亢進についても，分子生物学的検索が進んでいる[8]。

7) Igarashi M, et al：J Clin Invest 103：185-195, 1999.

8) Babaev VR, et al：J Clin Invest 103：1697-1705, 1999.

糖尿病の大規模臨床試験

糖尿病の管理は，虚血性心疾患の一次および二次予防の観点から重要である。その根拠となる大規模臨床試験について，一次予防と二次予防をわけて述べる。

一次予防

これまで行われた糖尿病患者への血糖降下介入試験を表**2**に示す。1978年に発表されたUGDP研究は，2型糖尿病1,027例を対象として，通常療法と強化療法に無作為に割り付けた初めての一次予防試験である[9]。心血管事故発症には有意差を認めなかったが，後に心血管事故の定義などの研究上の問題が指摘されて，現在，その結果に疑問がもたれている。その後，1型糖尿病1,441例を対象として，インスリンを1日2回まで使用する従来療法と，インスリンを1日3回以上使用する強化療法とで無作為比較試験を行ったDCCT研究が発表された[10]。この研究により，厳格な血糖コントロールで網膜症や腎症の発症進展抑制が可能であることが初めて証明された。しかし，対象が若年であり，また心血管系事故の予防効果には，有意差が認められなかった。さらに4,209例の2型糖尿病を15年間にわたって追跡して，食事療法と強化療法の無作為比較を行ったUKPDS研究が1998年に発表された[11]。SU薬ないしインスリンを用いた強化療法により，細小血管障害

9) University Group Diabetes Program：JAMA 240：37-42, 1978.

10) The Diabetes Control and Complications Trial Research Group：N Engl J Med 329：977-986, 1993.

11) UK Prospective Diabetes Study (UKPDS) Group：Lancet 352：837-853, 1998.

表1 AGEの動脈硬化進展機序

炎症促進作用	サイトカイン産生 単球－マクロファージ系走化因子産生
細胞増殖促進	単球および血管平滑筋からのPDGFなどの産生
内皮細胞	内皮細胞の透過性亢進 凝固活性促進作用（組織因子発現） 接着分子の発現増強 細胞内酸化ストレス増強作用（NFκB活性化）
細胞外マトリックス	膠原線維架橋形成 細胞外マトリックス産生増加 内皮下へのLDL捕捉 NO不活性化
リポ蛋白修飾	糖化LDL

（文献5より引用改変）

の予防効果が認められたものの，心筋梗塞発症抑制は16%の減少にとどまり，有意差は得られなかった。肥満を合併した2型糖尿病753例を対象として，食事療法と塩酸メトホルミンの効果を10年間追跡調査したUKPDSのサブ研究では，塩酸メトホルミン群での全死亡発生数は年間13.5/1,000人で，食事療法群の20.6人に比較して有意に低率であった(p=0.011)[12]。しかし，心筋梗塞発症抑制効果については，有意差は得られなかった(p=0.052)。塩酸メトホルミンは，1957年に開発されたインスリン抵抗性改善作用を有する2型糖尿病治療薬である。類薬であるフェンフォルミンが，乳酸アシドーシスをきたす副作用の問題で発売中止になったため，捨て去られていた薬物である。しかしUKPDS研究により，再び脚光を浴びて1995年に市場へ復活した異例の薬物である。塩酸メトホルミンを用いた複数の臨床試験からのメタアナリシスでは，塩酸メトホルミンとSU薬の血糖値とHbA1c改善効果は，同等であると報告されている。しかし，SU薬群では体重が増加したのに対して，塩酸メトホルミン群では逆に減少することが明らかにされた[13]。最近，塩酸メトホルミンによる脂肪肝改善効果などの全身的な代謝改善効果が脚光を浴びている[14]，さらに，ラットを用いた心筋虚血再灌流モデル実験では，塩酸メトホルミンが気絶心筋状態からの回復を促進することが報告され，心筋への直接効果にも期待が高まっている[15]。UKPDS研究は，現在も継続されており，2003年に発表される最終結果が待たれている。

12) UK Prospective Diabetes Study (UKPDS) Group : Lancet 352 : 854-865, 1998.

13) Johansen K : Diabetes Care 22 : 33-37, 1999.

14) Marchesini G, et al : Lancet 358 : 893-894, 2001.

15) Legtenberg RJ, et al : Horm Metab Res 34 : 182-185, 2002.

一方，耐糖能異常（impaired glucose tolerance；IGT）例への大規模介入試験も報告されている。3,819例のIGTを対象としたDPP研究では，週150分以上の運動などを盛り込んだ生活習慣改善強化療法群での3年後の糖尿病発症率は14%で，偽薬群の29%，メトホルミン群の22%と比較して有意に低値であったことが明らかにされた[16]。さらに，1,429例のIGTを対象として，αグルコシダーゼ阻害薬であるアカルボースの有効性を検証したSTOP-NIDDM研究が報告された。3年後

16) Diabetes Prevention Program Research Group : N Engl J Med 346 : 393-403, 2002.

表2　糖尿病への血糖低下介入試験と耐糖能異常への介入試験

DMへの血糖低下介入試験	実施年	対象	例数	結果
一次予防				
UGDP研究	1961～1975年（13年）	2型糖尿病	1,027例	心血管事故に有意差なし
VACSDM研究	1994～1996年（27ヵ月）	2型糖尿病	153例	心血管事故に有意差なし（減少傾向あり）
DCCT研究	1983～1993年（6.5年）	1型糖尿病	1,441例	心血管事故に有意差なし
UKPDS研究	1977～1997年（10年）	2型糖尿病	3,867例	心筋梗塞症が16%低下（有意差なし）
IGTへの介入試験	**実施年**	**対象**	**例数**	**結果**
DPP研究	1996～1999年（2.8年）	IGT	3,234例	生活改善強化が糖尿病発症を予防した ついでメトホルミンが糖尿病発症を予防した
STOP-NIDDM研究	1995～1998年（3.3年）	IGT	1,429例	アカルボース投与により糖尿病発症率が低下，正常耐糖能への回復率が高まった
NAVIGATOR研究	2001年から開始	IGT	未定	2008年に終了予定

の2型糖尿病発症率はアカルボース群で32%と，偽薬群の42%に比較して有意に低値であった。しかも，アカルボース群ではIGTから正常耐糖能への回復率も有意に高いことが示されて，注目を集めている[17]。また，7,500例のIGTを対象としたNAVIGATOR研究が2001年11月から進行中である。本研究は，ナテグリニドとバルサルタンによるIGTから2型糖尿病への進展予防および心血管疾患発症予防効果の検証を目的とし，2008年に終了予定である[18]。

17) Chiasson JL, et al : Lancet 359 : 2072-2077, 2002.

18) NAVIGATOR : Novartis press release.

二次予防

循環器内科医が日常診療で遭遇する糖尿病例は，すでに虚血性心疾患を発症しており，二次予防を要する例である。では，糖尿病を合併した虚血性心疾患例で血糖値を厳格にコントロールすれば，二次予防効果が得られるのであろうか。発症24時間以内の急性心筋梗塞症407例を対象として，急性期のグルコースインスリンカリウム(GIK)療法を検討した報告によると，GIK療法施行群の院内死亡率は5.2%で，対照群の15.2%と比較して有意に死亡率が低かったことが報告されている[19]。急性心筋梗塞症620例を対象としたDIGAMI研究では，急性期にインスリン静注療法を行い，その後にインスリン治療を行った群の1年後死亡率は8.6%と，通常治療群の18%に比較して有意に予後が良好であった($p=0.02$)[20]。特に，インスリン治療がなされていなかった糖尿病群での死亡率が52%も低下していた。

19) Diaz R, et al : Circulation 98 : 2227-2234, 1998.

20) Malmberg K, et al : J Am Coll Cardiol 26 : 57-65, 1995.

2,432例の心筋梗塞症患者を対象として7年間観察したフィンランドからの報告では，糖尿病合併例での心筋梗塞再発率は45%で，糖尿病のない例の18.8%に比較して2倍以上ときわめて高いことが示された[21]。しかし，現在わが国で進行中のJDCS（Japan Diabetic Complication Study）研究では，日本人の糖尿病例では，欧米で論じられているほど，心筋梗塞再発率が高くない可能性が示唆されており，わが国での糖尿病例での心筋梗塞再発率の調査も迫られている。

21) Haffner SM, et al : N Engl J Med 339 : 229-234, 1998.

一方，慢性の冠動脈疾患での厳格な血糖コントロールが予後を改善するかについての明確な根拠はなく，今後の検討が必要である。

糖尿病と冠動脈病変

冠動脈造影での検討によると，糖尿病例ではびまん性狭窄および多枝病変例が多いと報告されている。また最近では，血管内超音波検査や血管内視鏡を用いた検討もなされている。不安定狭心症55例を対象として冠動脈内視鏡で責任病変を観察した報告では，糖尿病での粥腫潰瘍形成が94%に観察され，非糖尿病例の60%と比較して有意に多いことが報告された。同様に血栓の観察頻度も糖尿病で有意に高く，マクロファージが組織因子の発現促進を増強していると考えられている[22]。さらに，一方向性アテレクトミーで切除した冠動脈硬化病変の病理学的検索によると，糖尿病例では大きな脂質核があり，マクロファージの発現が多いことが明らかにされた。さらに，血栓が非糖尿病例で40%のみに検出されたのに対して，糖尿病例では62%と有意に高く検出されたと報告されている[23]。

22) Silva JA, et al : Circulation 92 : 1731-1736, 1995.

23) Moreo PR, et al : Circulation 102 : 2180-2184, 2000.

糖尿病性心筋障害

近年，臨床研究や実験結果から，糖尿病性心筋障害とよばれる病態が明らかにされつつある。病理学的検索から，心筋内細動脈に増殖性変化があることが1960年に初めて報告された。その後1972年に，糖尿病剖検例で，細小血管障害，心筋肥大，心筋の融解，間質および血管周囲の線維化の存在が報告された[24]。さらに臨床例での検討から，糖尿病を合併した急性心筋梗塞症では，糖尿病のない例に比較して心不全になりやすいと報告されている[25]。その原因として，心外膜動脈病変とは別に，心筋内のmicro-angiopathyがかかわると考えられている。また，positron emission tomography（PET）での検討によって，ジピリダモールによる冠予備能が糖尿病例では低下していることが示されている[26]。以上から，糖尿病例では細小血管障害と冠予備能の低下から，非糖尿病例に比較して中等度の冠動脈狭窄でも心筋虚血を生じやすいと考えられている[27]。さらに，糖尿病性心筋障害例では，早期から左室拡張能異常を生じ，その後に収縮能障害が現れると報告されている。その機序として，筋原線維のCa-ATPase活性の低下による収縮蛋白や収縮調節蛋白の障害などの異常が指摘されている。この潜在性の拡張障害により心不全が生じやすいと考えられている。

24) Rubler S, et al : Am J Cardiol 30 : 595-602, 1972.

25) Stone HP, et al : J Am Coll Cardiol 14 : 49-57, 1989.

26) Pitkanen O, et al : Diabetes 47 : 248-255, 1998.

27) Henry P, et al : Am Heart J 134 : 1037-1048, 1997.

糖尿病例への冠血行再建術

多枝病変1,829例への冠血行再建術を検討したBARI研究では，糖尿病患者へのPCI施行7年後の生存率は55.7%で，冠動脈バイパス手術（coronary artery bypass grafting；CABG）施行群の76.4%に比較して，生命予後が有意に不良であることが明らかにされた[28]。また，糖尿病例ではPCI後の再狭窄率が高いことも示されている。現在のところ，糖尿病を有する例の多枝病変への血行再建術としては，CABGが推奨されている。種々のステントやカッティングバルーン，ロータブレータなどの新しい用具による再狭窄予防効果も期待されているが，いまだその効果は明らかではない。最近，再狭窄予防薬を吸着させた新しい冠動脈ステント（ラパマイシンステントなど）が開発され，欧米を中心に臨床応用が始まっている[29]。糖尿病例でも再狭窄が激減する可能性があり，注目されている。

28) The BARI investigators : J Am Coll Cardiol 35 : 1122-1129, 2000.

29) Morice MC, et al : N Engl J Med 346 : 1773-1780, 2002.

糖尿病の治療指針と心血管作動薬のevidence

糖尿病のコントロールは，単に血糖値を下げればいいというものではない。血糖をコントロールした結果が，冠動脈硬化の進展予防および生命予後改善につながらなくてはならない。糖尿病患者での冠動脈疾患予防のための血糖管理基準が日本糖尿病学会から示されている（表3）。また，心血管疾患発見の指針（表4：AHA，表5：当施設）および冠動脈疾患および血管疾患を有する糖尿病例への包括的リスク軽減指針が示されている（表6）。この指針では，糖尿病の血糖値を厳

ン分泌を回復させるような薬物の選択が望ましい。

表7　各種経口血糖降下薬の特徴(日本糖尿病学会編：糖尿病治療ガイド2000，糖尿病専門医研修ガイドブック，2001より)

■インスリン分泌刺激薬

a：スルホニル尿素系

膵β細胞上のSU受容体と結合して，インスリン分泌を促進して，血糖降下作用を示す。インスリン分泌能が比較的保たれて，食事運動療法での血糖コントロールが不十分な例が適応。

一般名	商品名	血中半減期	作用時間	剤型	1日投与量	代謝排泄
トルブタミド	ラスチノン，ジアベン	5.9時間	6～12時間	250mg，500mg	250～1500mg	腎
グリクロピラミド	デアメリンS	－	6時間	250mg	125～500mg	腎
アセトヘキサミド	ジメリン	3.2時間	10～16時間	250mg，500mg	250～500mg	腎
トラザミド	トリナーゼ	－	10～16時間	100mg，500mg	100～300mg	腎
クロルプロパミド	アベマイド	－	24～60時間	100mg，250mg	125～500mg	腎
グリブゾール	グルデアーゼ	－	12～24時間	125mg，250mg	125～500mg	腎
グリベンクラミド	オイグルコン，ダオニール	2.7時間	12～24時間	1.25mg，2.5mg	1.25～10mg	腎50％，肝50％
グリクラジド	グリミクロン	6～12時間	6～24時間	40mg	40～120mg	主に腎
グリメピリド	アマリール	1.5時間	6～12時間	1mg，3mg	1～4mg	腎40％，肝60％

b：フェニールアラニン誘導体

SU薬と同様に，膵β細胞上のSU受容体と結合して，インスリン分泌を促進して，血糖降下作用を示す。食事運動療法での血糖コントロールが不十分な軽症2型糖尿病が適応。

一般名	商品名	血中半減期	作用時間	剤型	1日投与量	代謝排泄
ナテグリニド	ファスティック，スターシス	0.8時間	3時間	30mg，90mg	270mg	腎肝

■ビグアナイド薬：肝での糖新生抑制，消化管からの糖吸収抑制，末梢でのインスリン抵抗性改善，食欲抑制あり。主に膵外作用で血糖降下作用を発揮する。若年から中年のインスリン非依存状態をもつ肥満を合併した症例が適応。

一般名	商品名	血中半減期	作用時間	剤型	1日投与量	代謝排泄
塩酸メトホルミン	グリコラン，メルビン	1.5～4.7時間	6～14時間	250mg	250～750mg	腎肝
塩酸ブホルミン	ジベトスB	3時間	6～14時間	50mg	50～150mg	腎

■αグルコシダーゼ阻害薬：小腸のαグルコシダーゼを阻害して，糖の消化を抑制して，吸収を遅らせることで，食後高血糖を抑制する。空腹時血糖が正常に近く，食後に200mg/dl以上の高血糖をきたす例が適応。

一般名	商品名	血中半減期	作用時間	剤型	1日投与量	代謝排泄
アカルボース	グルコバイ	不明	2～3時間	50mg，100mg	150～300mg	ほとんど吸収されず
ボクリボース	ベイスン	不明	2～3時間	0.2mg，0.3mg	0.6～0.9mg	ほとんど吸収されず

■チアゾジリン誘導体：インスリン抵抗性を改善して，血糖降下作用を示す。インスリン抵抗性の関与が強く，インスリン非依存性状態が適応。

一般名	商品名	血中半減期	作用時間	剤型	1日投与量	代謝排泄
ピオグリタゾン	アクトス	5時間	20時間	15mg，30mg	30mg	腎肝

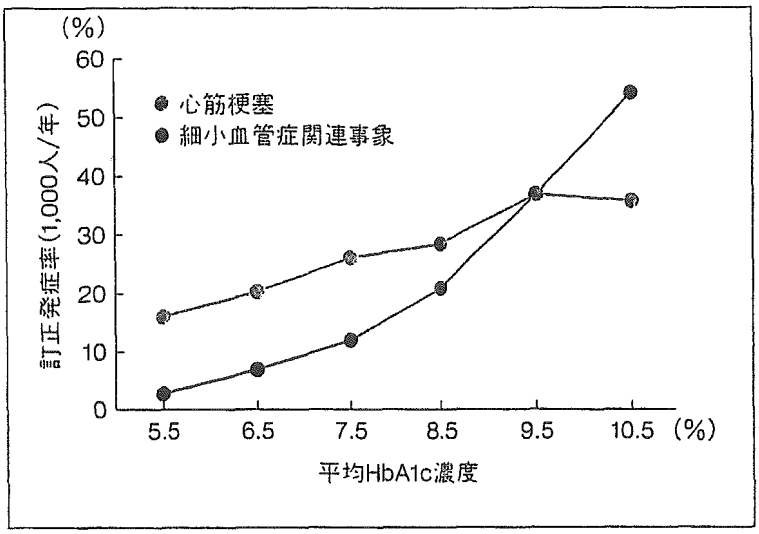


図2　糖尿病性血管合併症の発症と血糖コントロールの関連
HbAlcが上昇すると細小血管関連事象の発生頻度ほどではないが，心筋梗塞も発症率が高まる。さらにHbAlc値には閾値がなく，直線的に頻度が高まる。
(文献32より引用)

表8　糖尿病例へのβ遮断薬の効果

研究名，報告者	発表年	例数	観察期間	試験デザイン	結果
Norwegian multicenter Timolol研究	1983年	99例	17ヵ月	後ろ向き	心臓死の危険率が67%減少
Geteborg metoprolol研究	1988年	120例	3ヵ月	後ろ向き	再梗塞の危険率が76%減少
MIAMI研究	1989年	413例	15日	後ろ向き	心臓死の危険率が49%減少
Kjekshusら	1990年	268例	1年	後ろ向き	心臓死の危険率が67%減少
BIP研究	1996年	2,723例	3年	後ろ向き	心臓死の危険率が42%減少

表9　各種降圧薬の特徴

降圧薬	糖代謝への影響	総コレステロール	HDLコレステロール	中性脂肪
利尿薬	悪化させることがある	↑	↓→	↑
β遮断薬	悪化させることがある	→	↓	↑
α1遮断薬	改善	↓→	↑	↓→
ACE阻害薬	改善	↓→	→↑	↓→
ARB阻害薬	変化させないかやや改善	↓→	→	→
カルシウム拮抗薬	改善	→	→	→

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Overexpression of Interleukin-15 Protects against *Escherichia coli*–Induced Shock Accompanied by Inhibition of Tumor Necrosis Factor–α–Induced Apoptosis

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Interleukin (IL)–15, a potent inhibitor of tumor necrosis factor (TNF)–α–mediated apoptosis, causes multiple organ failure during endotoxic shock. We investigated the potential role of IL-15 in protection against *Escherichia coli*–induced shock by using IL-15 transgenic (Tg) mice. These mice were resistant to an otherwise lethal challenge with *E. coli*, although bacterial burden and serum levels of TNF-α were similar in non-Tg mice. Apoptosis in cells of the peritoneal cavity, liver, spleen, or lung was significantly suppressed in IL-15 Tg mice after *E. coli* infection. Peritoneal cells from naive IL-15 Tg mice were also resistant to TNF-α–induced apoptosis in vitro, and neutralization of endogenous IL-15 significantly aggravated TNF-α–induced apoptosis. Exogenous IL-15 prevented TNF-α–induced apoptosis in normal mice in vitro and improved the survival rate after *E. coli* challenge. These results suggest that IL-15 overexpression can prevent TNF-α–induced apoptosis and protect against *E. coli*–induced shock, indicating a possible therapeutic application of IL-15 for septic shock.

The incidence of infection with gram-negative bacteria such as *Escherichia coli* in patients undergoing abdominal surgery has increased in recent years. These infections frequently result in septic shock, which is caused by endotoxin/lipopolysaccharide (LPS) derived from gram-negative bacteria [1–3]. Mammals respond to LPS from gram-negative bacteria through toll-like receptor by producing a variety of immune cell mediators [4]. Among such mediators, tumor necrosis factor (TNF)–α and interleukin (IL)–1 are mainly responsible for triggering lethal shock [5, 6]. Multiple organ failure in patients who die of septic shock is characterized by lymphocyte and macrophage apoptosis throughout the body [7]. The apoptosis in septic shock appears to be triggered by signaling via members of the TNF receptor (TNFR) family [8–11], especially via TNF-αR type I, which induces activation of the Fas-associated death domain and caspase machinery. Caspase 3 is an especially important executioner molecule in the suicide cascade in LPS-induced apoptosis [12]. By use of a mouse cecal ligation and puncture model, it was re-

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All experiments complied with guidelines of the animal care and use committees of the authors' institutions and with US National Institutes of Health regulations on animal handling and usage.

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cently shown that caspase 3 inhibitors can improve survival in sepsis [13, 14]. This finding may contribute to the development of a therapeutic approach to control lethal shock caused by gram-negative bacteria.

IL-15, which belongs to the 4-helix bundle cytokine family [15–18] and uses β- and γ-chains of the IL-2 receptor (IL-2R) for signal transduction, has many properties that are the same as those of IL-2, even though it has no sequence homology with IL-2 [18–20]. In contrast to IL-2, which is produced mainly by activated T cells, IL-15 is produced by a wide variety of tissues, including placenta, skeletal muscle, kidney, and macrophages after stimulation with LPS [15, 18, 21]. IL-15 plays important roles in the development and maintenance of NK cells and various T cell subsets, including NK T cells and central memory CD8 T cells [20, 22–26]. IL-15 is a potent inhibitor of several apoptosis pathways in lymphocytes via induction of antiapoptotic molecules (e.g., Bcl-2) [27, 28] and it blocks TNFR1-mediated cell death of fibroblasts by inhibition of a very early step in the apoptosis signaling cascade [29]. Thus, IL-15 may be useful as a powerful apoptosis antagonist for prevention of multiple organ failure with apoptosis caused by septic shock. Our goal in this study was to determine the ability of IL-15 to protect against septic shock caused by gram-negative bacteria. We examined the susceptibility of IL-15 transgenic (Tg) mice to infection with a high dose of *E. coli*.

MATERIALS AND METHODS

Animals. C57BL/6-background IL-15 Tg mice, which were constructed by using IL-15 cDNA under the control of a major histocompatibility complex class I promoter, have been described elsewhere [26]. In brief, full-length cDNA encoding the murine IL-15 gene with exon 5 (710 bp) was cloned between the *Bam*HI and *Sal*I sites of a Tg expression vector, pHSE-3′, which contains the H2-K promoter, the mouse immunoglobulin enhancer, the mouse β-globulin splice site, and poly A signal. Transgene DNAs were microinjected into the male pronucleus of fertilized single-cell embryos of C57BL/6 mice. Microinjected eggs were transferred to pseudopregnant C57BL/6 foster mothers. IL-15 Tg mice were identified by digesting genomic DNA with *Pst*I, followed by Southern blot analysis with an IL-15–specific probe. In each experiment, age- and sex-matched C57BL/6 mice (Charles River Japan) were used as controls. Mice were maintained under specific pathogen–free conditions and were offered food and water ad libitum. At the time of the study, all mice were 6–8 weeks old.

Microorganisms. *E. coli* (ATCC no. 26) was grown in a brain-heart infusion broth (Difco Laboratories), washed repeatedly, resuspended in PBS, and stored at −70°C in small aliquots until use. The concentration of bacteria was quantified by plate counts.

Antibodies and reagents. Fluorescein isothiocyanate (FITC)–conjugated active caspase 3 monoclonal antibody (MAb), FITC-conjugated anti–Bcl-2 MAb, phycoerythrin (PE)–conjugated anti-CD11b MAb (M1/70), biotin-conjugated anti-Ly6G (Gr-1) MAb (RB6-8C5), and streptavidin-conjugated Cy-Chrome MAb were purchased from PharMingen. We purchased FITC-conjugated anti–annexin V MAb (145-2C11) from Sigma Chemical and obtained 2.4G2 (anti–FcRII/III-specific MAb, rat IgG1, producing hybridoma) from American Type Culture Collection. Murine recombinant (r) IL-15 was obtained from Research Diagnostics.

Soluble IL-15Rα (sIL-15Rα) preparation. sIL-15Rα was generated as described elsewhere [30, 31]. The cDNA for the extracellular domain of IL-15Rα or of the mutant IL-15Rα, as a binding control, was amplified by reverse-transcriptase polymerase chain reaction from total RNA isolated from J774.1. The purified amplified products were confirmed by sequencing, digested with *Eco*RI and *Bam*HI, and ligated into a similarly digested pGEX-5X-2 procaryotic expression vector, according to the manufacturer's instructions (Amersham Pharmacia Biotech). We used the recombinant plasmid to transform competent *E. coli* BL21 cells (Stratagene) to produce the glutathione *S*-transferase IL-15Rα fusion protein. The soluble fusion protein was purified by using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). LPS was not detected in the purified sIL-15Rα preparation by the limulus amebocyte test (<0.01 ng/mg; E-Toxate; Sigma). The optimal sIL-15Rα concentration was determined based on the finding that 10 ng/mL sIL-15Rα blocked bioactivity of 0.1 ng/mL IL-15 in a CTLL2 cell line assay [30].

Cell preparation. Blood was obtained by retro-orbital plexus puncture at 1, 3, 6, and 12 h after *E. coli* challenge, and serum levels of various cytokines were determined at these time points. Then peritoneal exudate cells (PECs) were obtained by lavage of the peritoneal cavity with 5 mL of Hanks' balanced salt solution (HBSS) after the injection. PECs were prepared by centrifugation and resuspended in RPMI 1640 containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 m*M* HEPES. The cells were plated and allowed to adhere for 2 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Nonadherent cells were removed, and adherent cells were washed several times with HBSS. Adherent cells were collected by scraping with a rubber policeman, washed, and counted. More than 95% of the cells obtained by this procedure were macrophages.

Bacterial growth in organs. After infection, peritoneal exudates were obtained from the peritoneal cavity by lavage with 5 mL of HBSS. Serial dilutions of the exudate samples were plated to determine the number of viable bacteria. For enumeration of viable bacteria in the liver, the liver was perfused with 8 mL of sterile HBSS to wash out bacteria in the blood

vessels immediately after the mice were bled. Livers and spleen were removed and separately placed in sterile Teflon-coated homogenizers (Asahi Techno Glass), each containing 5 mL of cold PBS. After each organ was thoroughly homogenized, the bacterial counts in the homogenates were established by plating serial 10-fold dilutions in sterile distilled water on tryptic soy agar (Nissui Laboratories). Colonies were counted after incubation for 24 h at 37°C.

Cytokine assays. Serum cytokine levels were determined by ELISAs. ELISAs for TNF-α, IL-10, IL-12 p40, IL-1β, interferon (IFN)-γ, and IL-4 were performed by using MAbs according to the manufacturer's instructions (Genzyme). An ELISA for IL-18 was performed by using MAb MBL according to the manufacturer's instructions (Medical & Biological Laboratories).

Staining of PECs. We stained PECs by the Pappenheim staining method with modified May-Gruenwald's solution (Merck) and Giemsa stain solution (Katayama Chemical). Cells were then divided into neutrophils, macrophages, and lymphocytes by standard morphology. At least 200 cells were counted, and the absolute number of each cell type was calculated.

Flow cytometric analysis. Plastic adherent PECs were preincubated with a culture supernatant from 2.4G2 to prevent nonspecific staining. For the annexin V staining assay, the cells were then stained with anti-CD11b MAb coupled to PE and biotinylated (BN) anti-Gr-1 MAb. The anti-Gr-1 BN MAb staining was revealed by streptavidin-conjugated Cy-Chrome. After the cells were washed with HBSS and incubated with 5 μL of FITC-conjugated annexin V for 15 min at room temperature in the dark, we added 400 μL of 1× binding buffer, as recommended by the manufacturer (Sigma Chemical). For propidium-iodide (PI) staining, the cells were washed twice with PBS and fixed with ice-cold 70% ethanol/PBS. These cells were kept on ice for $\geq$1 h. Subsequently, the medium was removed by centrifugation, and the pellets were resuspended in 100 μL of PBS. The cells were then incubated in the dark for 30 min at 4°C in the presence of 50 μg/mL PI (Sigma) and 250 μg/mL DNase-free RNase A (Roche). Thereafter, cell cycle status and apoptosis were determined by flow cytometer (FACSCalibur; Becton Dickinson).

For active caspase 3-specific staining, the cells were stained with Cy-Chrome anti–Gr-1 and PE-CD11b, fixed and permeabilized with CytoPerm/Cytofix, and stained with FITC-conjugated anti–active caspase 3. For intracellular Bcl-2 staining, the cells were surface stained as described above. After being washed, the cells were stained intracellularly for Bcl-2 by using FITC-conjugated hamster anti–mouse Bcl-2 antibody (clone 3F11) or its isotype control antibody (hamster IgG), according to the manufacturer's instructions (PharMingen). The cells were then analyzed by flow cytometer (FACSCalibur). Live cells were carefully gated by forward and side scattering. Data were analyzed with CellQuest software (BD Biosciences).

Histopathologic examination. Liver, spleen, kidneys, and lungs were removed and fixed in 10% buffered formalin. After embedding in paraffin, 3-μm tissue sections were cut and stained with hematoxylin-eosin for morphologic evaluation. We performed the TUNEL (in situ terminal dUTP nick-end-labeling) assay with an in situ apoptosis detection kit (Apoptag; Intergen), according to the manufacturer's instructions. In brief, paraffin-embedded sections were deparaffinized and rehydrated and then permeabilized by incubation with 20 mg/mL proteinase K (Sigma) for 15 min at 37°C. The tissue sections were then washed in distilled water. Inactivation of endogenous peroxidases was accomplished by immersing the tissue sections in 3% hydrogen peroxide diluted in methanol for 10 min at room temperature. After the slides were washed with distilled water, the sections were incubated in equilibration buffer for 5 min. The sections were then incubated with the labeling solution containing terminal deoxynucleotidyl transferase in a humidified chamber for 1 h at 37°C. Reactions were terminated by rinsing the sections in a stop-wash buffer. The incorporated digitonigen-dUTP was detected by incubation with antidigoxigenin peroxidase at room temperature for 30 min; positive reactions were revealed by use of 3,3′ diaminobenzidine. Methyl green was used for counterstaining for nuclei, and slides were coverslipped.

Statistical analysis. Data were analyzed by Student's *t* test; survival data were analyzed by log-rank test. Between-mean comparisons were done by Wilcoxon test. We considered $P < .05$ to be statistically significant.

RESULTS

***IL-15 Tg mice are resistant to lethal infection with* E. coli.** We first examined the survival rate of IL-15 Tg mice after infection with *E. coli*. A high dose of *E. coli* (10^9 cfu/mouse; $2 \times LD_{50}$ to non-Tg mice) was injected intraperitoneally (ip) into IL-15 Tg or non-Tg mice. Non-Tg mice began to die 8 h after the injection. More than 85% of the non-Tg mice died within 12 h after the injection, whereas all IL-15 Tg mice survived >2 weeks (figure 1; $P < .01$).

We next compared numbers of bacteria in various organs of non-Tg mice and IL-15 Tg mice after *E. coli* injection. The numbers of bacteria in organs in IL-15 Tg mice were the same as in non-Tg mice after *E. coli* injection at both high and low doses (figure 2*A*). These results suggest that improved survival of IL-15 Tg mice is not due to differences in bacterial burden.

Infection with a high dose of *E. coli* frequently results in endotoxic shock, which is caused mainly by inflammatory cytokines, such as TNF-α and IL-1 [5]. Therefore, we next examined serum levels of proinflammatory cytokines (TNF-α, IL-12, IL-1β, and IL-18) and the serum level of an anti-inflammatory cytokine, IL-10, in IL-15 Tg and non-Tg mice af-

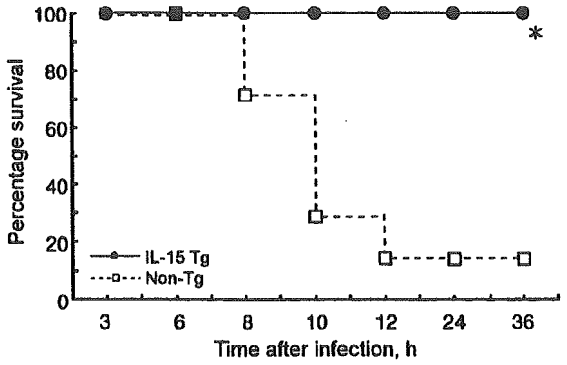

Figure 1. Survival curves of interleukin (IL)–15 transgenic (Tg) and non-Tg mice after *Escherichia coli* infection. IL-15 Tg and age-matched non-Tg mice were injected intraperitoneally with 10^9 cfu of *E. coli* each ($2 \times LD_{50}$). Survival was monitored for 48 h ($n = 7$–10 mice/group in each experiment). Results shown are typical of 1 of 3 independent experiments. Difference between survival curves was statistically significant. $^*P < .01$ (log-rank test).

ter ip inoculation with *E. coli* at a dose of 10^8 cfu/mouse ($0.2 \times LD_{50}$ to non-Tg mice). As shown in figure 2*B*, serum TNF-α and IL-10 levels were at maximum levels 1 h after *E. coli* injection. IL-12 p40 and IL-1β levels in serum were at maximum levels 3 h after the injection, and the IL-18 level peaked 12 h after the injection in both IL-15 Tg and non-Tg mice. There were no significant differences between TNF-α and IL-1β levels in IL-15 Tg and non-Tg mice. Although serum levels of IL-10 and IL-12p40 tended to increase and decrease, respectively, in IL-15 Tg mice, the levels in IL-15 Tg mice were not statistically different from those in non-Tg mice. Serum IL-4 and IFN-γ were not detected in either IL-15 Tg or non-Tg mice at any stage after *E. coli* injection (data not shown). These results suggest that improvement in survival of IL-15 Tg mice is not due to suppression of production of cytokines that induce endotoxic shock.

Impairment of programmed cell death in IL-15 Tg mice after* E. coli *infection. We next examined host cellular response to *E. coli* by assessing the kinetics of PECs after *E. coli* infection. PECs were obtained at indicated times after injection of a high dose of *E. coli* (10^9 cfu) and stained by the Pappenheim staining method for light microscopy examination. We calculated the absolute number of cells of each type by multiplying the total numbers by the percentages as assessed by morphologic findings. The absolute numbers of macrophages were significantly greater in IL-15 Tg mice than in non-Tg mice 3 and 6 h after *E. coli* injection (figure 3*A*; $P < .05$).

A high dose of LPS often induces programmed cell death in various cells, especially macrophages, by apoptosis via TNFR-mediated death signaling [32]. Therefore, we next examined the frequency of programmed cell death in PECs by using the annexin V staining method after injection of 10^9 cfu of *E. coli*. PECs obtained from the non-Tg and IL-15 Tg mice 6 h after injection of 10^9 cfu of *E. coli* were stained with anti-CD11b (Mac-1), anti–Gr-1, and anti-annexin V MAbs. Analysis gates were set on $CD11b^+Gr\text{-}1^+$ cells (corresponding to neutrophils), $CD11b^+Gr\text{-}1^-$ cells (corresponding to macrophages), and $CD11b^-Gr\text{-}1^-$ cells (corresponding to lymphocytes). The annexin V levels of the gated populations are shown as single

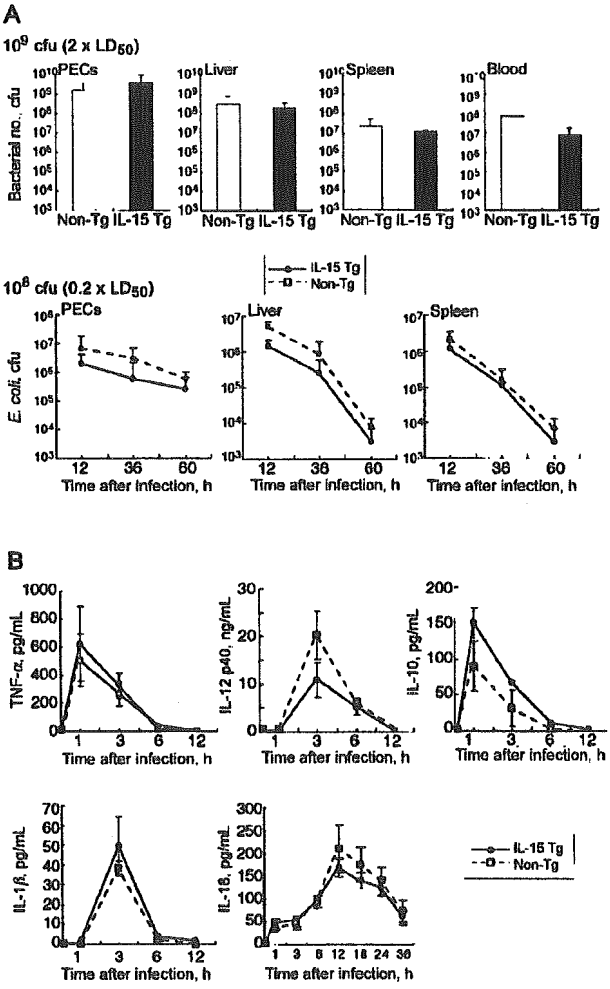

Figure 2. Bacterial growth and cytokine production in interleukin (IL)–15 transgenic (Tg) and non-Tg mice after *Escherichia coli* infection. *A*, IL-15 Tg and age-matched non-Tg mice were injected intraperitoneally with 10^9 cfu of *E. coli* each ($2 \times LD_{50}$; *upper panel*) or 10^8 cfu of *E. coli* each ($0.2 \times LD_{50}$; *lower panel*). No. of bacteria recovered from the peritoneal cavity (peritoneal exudate cells; PECs), liver, spleen, and blood of infected mice was determined 6 h after injection *(upper panel)* or at indicated times after *E. coli* injection *(lower panel)*. *B*, Tumor necrosis factor (TNF)–α, IL-12 p40, IL-10, IL-1β, and IL-18 levels in serum were determined by ELISA. Data are from $\geq$3 separate experiments and are expressed as mean $\pm$ SD of 5 mice from a representative experiment. No statistically significant differences were found between IL-15 Tg and non-Tg mice.

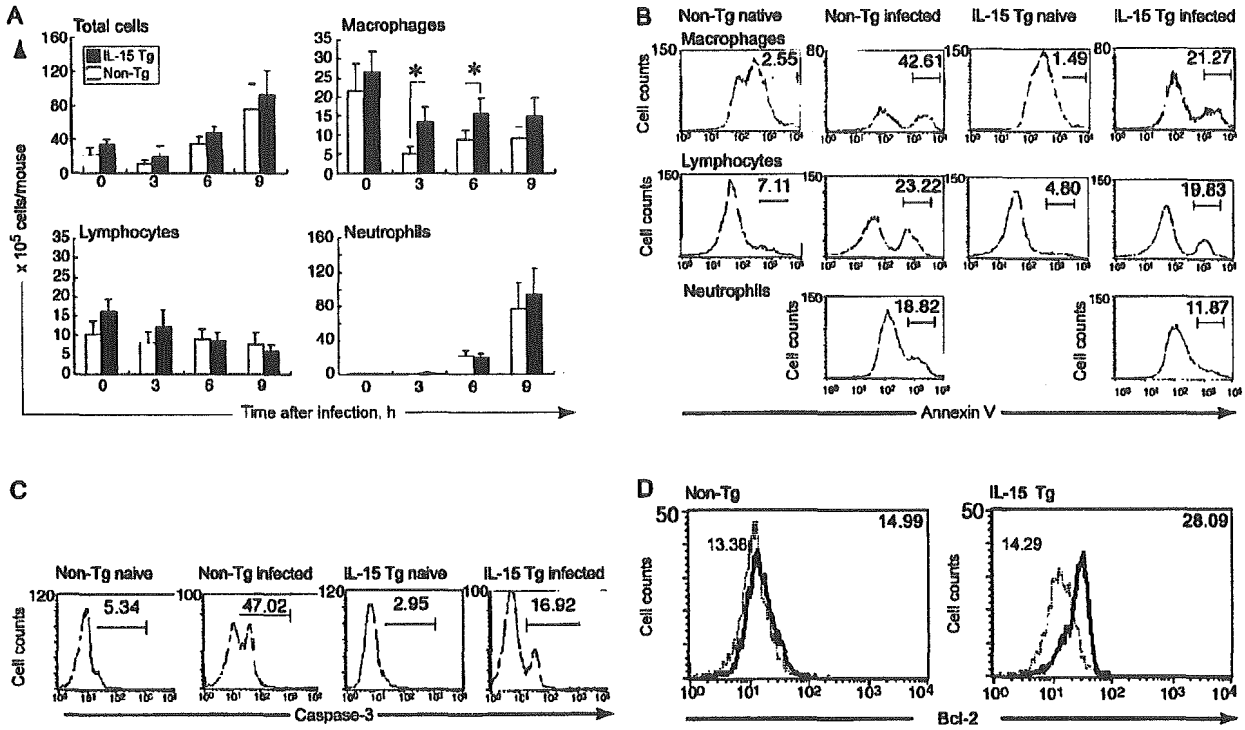

Figure 3. Impairment of programmed cell death in interleukin (IL)–15 transgenic (Tg) mice after *Escherichia coli* infection. *A*, IL-15 Tg and non-Tg mice were inoculated with 10^9 cfu of *E. coli* each. Peritoneal exudate cells (PECs) were obtained at indicated times after infection. Absolute nos. of cells of each type were calculated by multiplying total nos. by the percentages, as assessed by morphologic findings. Data are shown as mean ± SD. *$P < .05$. *B*, Flow cytometric analysis of peritoneal cells stained with annexin V. PECs from non-Tg and IL-15 Tg mice 6 h after injection with 10^9 cfu of *E. coli* were stained with anti-CD11b (Mac-1), anti–Gr-1, and anti–annexin V monoclonal antibody (MAb). Analysis gates were set on CD11b$^+$ Gr-1$^+$ cells (corresponding to neutrophils), CD11b$^+$Gr-1$^-$ cells (corresponding to macrophages), and CD11b$^-$ Gr-1$^-$ cells (corresponding to lymphocytes). Annexin V levels of the gated populations are shown as single-color histograms. Percentage of annexin V–positive cells is indicated. *C*, Flow cytometric analysis of active caspase 3 expression in peritoneal adherent cells after *E. coli* infection. Cells were similarly surface stained, stained with intracellular active caspase 3, and analyzed by flow cytometry. *D*, Flow cytometric analysis of Bcl-2 expression in peritoneal adherent cells after *E. coli* infection. PECs from non-Tg and IL-15 Tg mice 6 h after injection with 10^9 cfu of *E. coli* were stained with anti-CD11b, anti–Gr-1, and anti–Bcl-2 MAbs. Analysis gates were set on CD11b$^+$ Gr-1$^-$ cells. The Bcl-2 levels of the gated populations are shown as single histograms; no. in the upper right corner indicates the mean fluorescence intensity. The Bcl-2 levels in cells from naive mice are overlaid on each histogram as a dashed line; mean fluorescence intensity is indicated by the value in the upper left corner of the plot. Data are representative of 3 independent experiments.

histograms. Figure 3*B* shows typical results, and table 1 summarizes data of 3 independent experiments. A major increase in the percentage of peritoneal macrophages positive for annexin V was observed in non-Tg mice 6 h after *E. coli* injection (figure 3*B*), but there were significantly fewer apoptotic cells in IL-15 Tg mice than in non-Tg mice after the injection (figure 3*B*). Although the increases in annexin-positive apoptotic cells in neutrophils and lymphocytes seemed to be impaired in IL-15 Tg mice following *E. coli* injection, the numbers did not differ statistically from those in non-Tg mice.

The caspase 3 proenzyme becomes cleaved into its active form during apoptosis and is thus a good marker for programmed cell death [33, 34]. Therefore, we next examined active caspase 3 expression by flow cytometry, to determine whether peritoneal cells were dying by caspase-dependent apoptosis. Peritoneal adherent cells isolated from naive and *E. coli*–infected mice were stained as described above. These cells were also stained intracellularly with an antibody specific for active caspase 3. As shown in figure 3*C*, there was a significant

Table 1. Annexin V expression in peritoneal adherent cells after *Escherichia coli* infection of naive or infected mice.

Mouse strain	Naive	Infected
Non-Tg	7.32 ± 4.92	40.07 ± 4.55
IL-15 Tg	6.92 ± 5.06	21.83 ± 0.51[a]

NOTE. Percentage of annexin V–positive cells is shown. Data are mean ± SD of 3 individual experiments. IL, interleukin; Tg, transgenic.

[a] $P < .05$, vs. values for non-Tg mice (Student's *t* test).

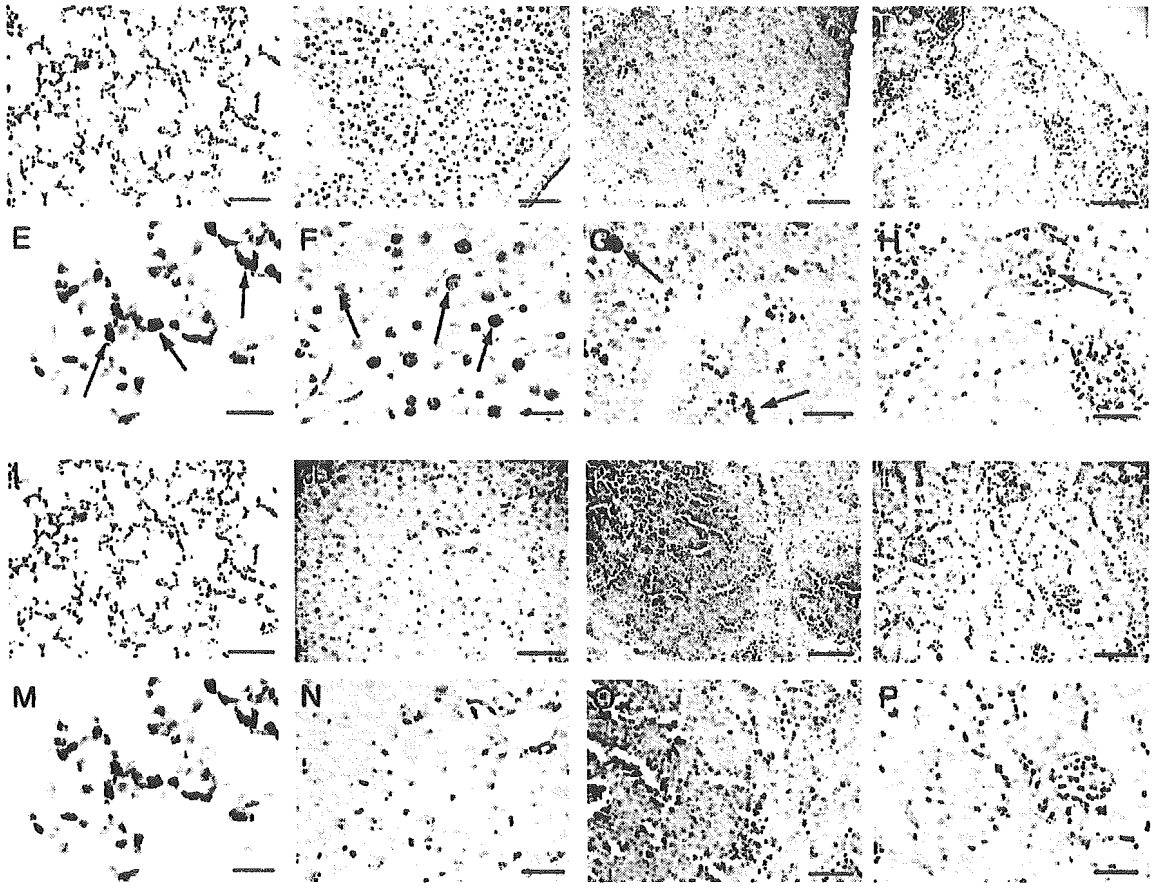

Figure 4. Apoptotic cells in situ in organs after *Escherichia coli* infection. DNA fragmentation in lung *(A, E, I,* and *M),* liver *(B, F, J,* and *N),* spleen *(C, G, K,* and *O),* and kidney *(D, H, L,* and *P),* of *E. coli*–infected non-transgenic (Tg) mice *(A–H)* and interleukin (IL)–15 Tg mice *(I–P)* (10^9 cfu/mouse) was detected by the TUNEL (in situ terminal dUTP nick-end-labeling) method (Materials and Methods). TUNEL-positive cells are dark brown *(arrows).* TUNEL staining revealed more apoptotic cells in the lung, liver, and spleen of non-Tg mice than in IL-15 Tg mice *(arrows). A–H,* non-Tg mice; *I-P,* IL-15 Tg mice. *Bars: A–D* and *I–L,* 200 μm; *E–H* and *M–P,* 100 μm. All data are representative of 3 independent experiments.

increase in active caspase 3–specific staining in the peritoneal cells from *E. coli*–infected non-Tg mice, compared with cells of IL-15 Tg mice. These results suggest that *E. coli* infection directly resulted in rapid death by apoptosis in peritoneal adherent cells and that those in IL-15 Tg mice were resistant to *E. coli*–induced apoptosis.

One of the most widely studied regulators of cell death is the Bcl-2 superfamily. Bcl-2 plays a critical role in regulation of cell survival and apoptosis by preventing release of cytochrome *c* after mitochondrial damage [35, 36]. IL-15 increases Bcl-2 via c-myc and STAT 5 activation. Susceptibility to TNFR-mediated apoptosis is related to Bcl-2 expression [28]. Therefore, we compared the expression levels of Bcl-2 at the protein level in peritoneal adherent cells obtained from non-Tg and IL-15 Tg mice 6 h after injection of 10^9 cfu of *E. coli.* Figure 3*D* shows a result typical of 3 independent experiments. The Bcl-2 expression levels in cells obtained from IL-15 Tg mice before infection were almost the same as those of non-Tg mice. However, the expression level in the peritoneal cells obtained from IL-15 Tg mice after injection was significantly higher than in non-Tg mice.

E. coli *infection induces a marked increase in apoptotic cells in non-Tg mouse organs.* For detection of apoptosis in situ, liver, spleen, kidney, and lung tissues of *E. coli*–infected IL-15 Tg and non-Tg mice were obtained 12 h after infection. Apoptotic cells were identified by the TUNEL technique. After *E. coli* infection, we observed more cells undergoing apoptosis in lung, liver, and spleen of non-Tg mice. The apoptotic cells showed characteristic shrinking, chromatin clumping, and nuclear fragmentation. These apoptotic cells were both localized in foci and scattered. Most of the events in the liver were in cells lining the sinusoids; those in the spleen were in the germinal centers and the marginal zone. In contrast, apoptotic cells remained low in IL-15 Tg mice. There were few apoptotic

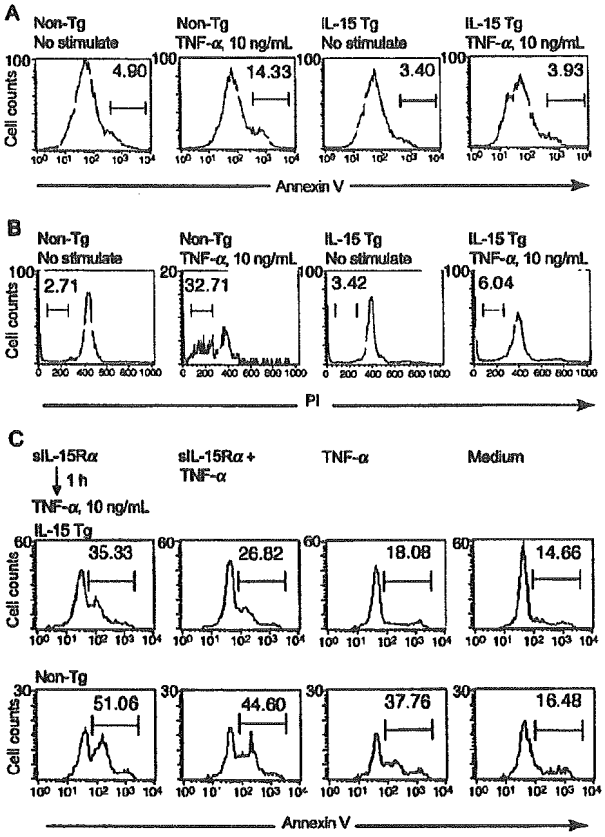

Figure 5. Flow cytometry shows that peritoneal cells from naive interleukin (IL)–15 transgenic (Tg) mice are resistant to tumor necrosis factor (TNF)–α–induced apoptosis. *A*, Peritoneal adherent cells (10^6 cells/mL) from naive non-Tg and naive IL-15 Tg mice were incubated in the presence or absence of TNF-α (10 ng/mL) for 12 h, collected, and stained with annexin V, as recommended by the manufacturer (Sigma). *B*, Assessment of DNA cleavage by propidium iodide (PI) staining. Peritoneal adherent cells from naive non-Tg and naive IL-15 Tg mice were incubated for 24 h in the presence or absence of TNF-α (10 ng/mL). Cells were collected, stained with PI, and analyzed by flow cytometry. Apoptosis was judged by the presence of cells with hypodiploid DNA content after cells were exposed to TNF-α. *C*, Percentage of cells in which apoptosis was induced by TNF-α treatment was increased by neutralization of endogenous IL-15. Peritoneal adherent cells from naive IL-15 Tg and naive non-Tg mice were treated with soluble (s) IL-15Rα (10 ng/mL) and with TNF-α (10 ng/mL) 1 h later, sIL-15Rα plus TNF-α, TNF-α (10 ng/mL), or medium. After incubation for 12 h, cells were collected and stained with annexin V. *B* and *C*, Data are representative of 3 independent experiments.

cells in kidney tissue after *E. coli* infection in both IL-15 Tg and non-Tg mice. These results suggest that *E. coli* infection induced significantly more apoptosis of cells in systemic organs in non-Tg mice than in IL-15 Tg mice (figure 4).

Peritoneal cells from naive IL-15 Tg mice are resistant to TNF-α–induced apoptosis. We performed experiments to determine whether cells from IL-15 Tg mice are resistant to TNF-α–mediated apoptosis. Peritoneal adherent cells were obtained from naive IL-15 Tg and naive non-Tg mice, as described above, incubated in the presence or absence of TNF-α (10 ng/mL) for 12 h, collected, and stained with annexin V, as recommended by the manufacturer (Sigma Chemical). A major increase in the percentage of peritoneal cells positive for annexin V was found in non-Tg mice after TNF-α stimulation (figure 5*A*), whereas apoptosis was impaired in IL-15 Tg mice, compared with that in non-Tg mice after TNF-α stimulation (figure 5*A*).

We did additional experiments to examine the cell cycle. Peritoneal adherent cells from naive IL-15 Tg and naive non-Tg mice were incubated in the presence or absence of TNF-α (10 ng/mL) for 24 h and stained with PI, as described above. Flow cytometric analysis of PI-stained cells demonstrated TNF-α–induced apoptosis in peritoneal cells, as judged by the presence of cells with hypodiploid DNA content. As shown in figure 5*B*, 24 h of exposure to TNF-α caused marked apoptosis in the cells from non-Tg mice (figure 5*B*); cells from IL-15 Tg mice showed little apoptosis, compared with that in cells from non-Tg mice after TNF-α treatment (figure 5*B*). Table 2 summarizes data from 3 independent experiments.

To determine whether endogenous IL-15 production plays an important role in protection against TNF-α–induced apoptosis in IL-15 Tg mice, we examined the effect of neutralization of endogenous IL-15 by soluble IL-15Rα on TNF-α–induced apoptosis in peritoneal adherent cells. Results showed that more than 10 ng/mL soluble IL-15Rα inhibited IL-15–induced proliferation of CTLL-2 (data not shown). Figure 5*C* shows that addition of soluble IL-15Rα resulted in an increased percentage of cells from IL-15 Tg mice in which TNF-α–induced apoptosis occurred. Of note, the percentage of peritoneal cells from non-Tg mice in which apoptosis was induced by TNF-α treatment was also increased by neutralization of endogenous IL-15. These results suggest that endogenous IL-15 plays an important role in TNF-α–induced apoptosis in IL-15 Tg mice and in normal mice.

Exogenous IL-15 rescues peritoneal cells from TNF-α–induced apoptosis and protects against* E. coli*–induced lethal

Table 2. Expression of annexin V and propidium iodide in tumor necrosis factor (TNF)–α–induced apoptosis.

Mouse strain	Annexin V		Propidium iodide	
	None	TNF-α	None	TNF-α
Non-Tg	4.9 ± 1.01	12.28 ± 2.89	6.76 ± 5.72	35.4 ± 3.79
IL-15 Tg	3.4 ± 0.89	5.78 ± 2.61[a]	2.65 ± 1.08	5.5 ± 0.74[b]

NOTE. Percentage of apoptotic cells is shown. Data are mean ± SD of 3 individual experiments. IL, interleukin; Tg, transgenic.

[a] $P < .05$, vs. values for non-Tg mice (Student's *t* test).

[b] $P < .01$, vs. values for non-Tg mice (Student's *t* test).

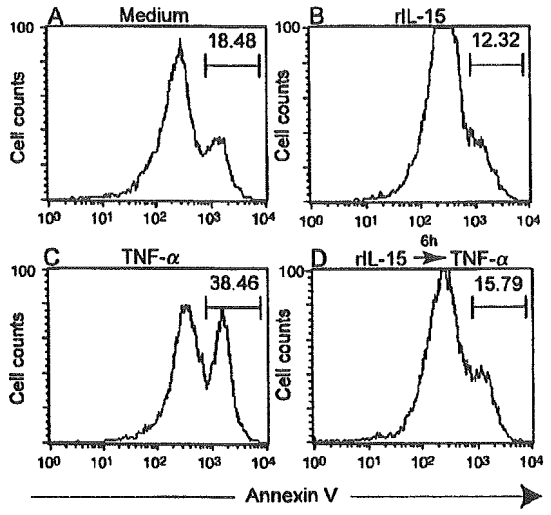

Figure 6. Effect of recombinant (r) interleukin (IL)–15 on tumor necrosis factor (TNF)–α–induced apoptosis. Peritoneal adherent cells from naive C57BL/6 mice were treated (all quantities: 10 ng/mL) with medium *(A)*, rIL-15 *(B)*, TNF-α *(C)*, or rIL-15 and then with TNF-α 6 h later *(D)*. After incubation for 12 h, cells were collected and stained with annexin V. Apoptosis was analyzed by flow cytometry. Data are representative of 3 independent experiments.

shock. To determine whether exogenous IL-15 is useful for protection against multiple organ failure with apoptosis after lethal infection with *E. coli*, we first examined the effects of IL-15 stimulation in vitro on TNF-α–induced apoptosis. Peritoneal adherent cells from naive normal mice were treated for 12 h with medium, 10 ng/mL rIL-15, 10 ng/mL TNF-α, or with rIL-15 and then with TNF-α 6 h later. After incubation for 12 h, the cells were collected and stained with annexin V, and apoptosis was analyzed by flow cytometry.

Figure 6 shows that coincubation with rIL-15 rendered peritoneal cells resistant to apoptosis induced by TNF-α. Peritoneal adherent cells treated with TNF-α alone showed extensive apoptosis 12 h after treatment (figure 6*C*), whereas, in cells treated with rIL-15 and then with TNF-α, TNF-α–induced apoptosis by IL-15 was suppressed (figure 6*D*). Table 3 summarizes data from 3 independent experiments. These data suggest that IL-15 blocks TNF-α–induced apoptosis in peritoneal cells at the signaling cascade stage.

We next examined the effects of in vivo administration of rIL-15 on protection against infection with a lethal dose of *E. coli* injected ip in normal mice. rIL-15 (1 or 10 μg) or PBS for control was injected ip immediately after an ip inoculation of 10^9 cfu of *E. coli*. As shown in figure 7, 80% of PBS-treated mice died within 24 h after injection, whereas 30% of the mice given 1 μg of rIL-15 and 60% of the mice given 10 μg of rIL-15 survived >24 h after injection ($P < .05$, generalized Wilcoxon test). Thus, in vivo administration of rIL-15 improved the survival rate of mice infected with *E. coli*.

DISCUSSION

In the present study, we demonstrated that IL-15 Tg mice have resistance against lethal infection with a high dose of *E. coli* accompanied by impaired programmed cell death in the peritoneal cavity, liver, spleen, and lung. Peritoneal adherent cells from naive IL-15 Tg mice exhibited resistance to TNF-α–induced apoptosis, the rate of which was increased by neutralization of endogenous IL-15. These results suggest that IL-15 overexpression can protect against septic shock via inhibition of apoptosis in host cells. This speculation was supported by results that showed that the addition of exogenous IL-15 in vitro inhibited TNF-α–induced apoptosis and that in vivo administration of exogenous IL-15 protected normal mice from lethal infection with *E. coli*. Thus, administration of IL-15 could be useful as a new immunotherapeutic approach for controlling septic shock.

Multiple organ failure in patients who die of sepsis is characterized by apoptosis in lymphocytes and macrophages throughout the body [7]. Several lines of evidence in animal models indicate that sepsis induces extensive lymphocyte and macrophage apoptosis, which is triggered by signaling via TNFR [14, 37–39]. Inhibition of TNFR signaling by soluble TNFR prevents lethal shock caused by LPS [40], and direct inhibition of the downstream region of the apoptosis cascade by a caspase 3 inhibitor can improve the survival rate of animals with sepsis induced by cecal ligation and puncture [13, 14]. In the lethal model of sepsis, prevention of lymphocyte cell death was associated with a marked reduction in blood bacterial counts and inflammatory cytokines, such as TNF-α and IL-1β [14]. Hotchkiss et al. [14] concluded that caspase inhibitors enhance immunity by preventing lymphocyte apoptosis and reduce the amounts of effector molecules for lethal shock, resulting in improvement in survival rate.

In our lethal model of sepsis, the levels of bacterial burden and inflammatory cytokine production in IL-15 Tg mice were similar to those in non-Tg mice after *E. coli* infection. These results suggest that susceptibility of IL-15 Tg mice to mediators that trigger endotoxin shock is less than that of non-Tg mice.

Table 3. Effect of recombinant interleukin (IL)–15 on tumor necrosis factor (TNF)–α–induced apoptosis (annexin V).

Medium	IL-15	TNF-α	IL-15 to TNF-α
18.48 ± 3.62	16.3 ± 5.62	32.62 ± 8.26	16.22 ± 0.60[a]

NOTE. Percentage of annexin V–positive cells is shown. Data are mean ± SD of 3 individual experiments.

[a] $P < .05$, vs. values for TNF-α–stimulated group (Student's *t* test).

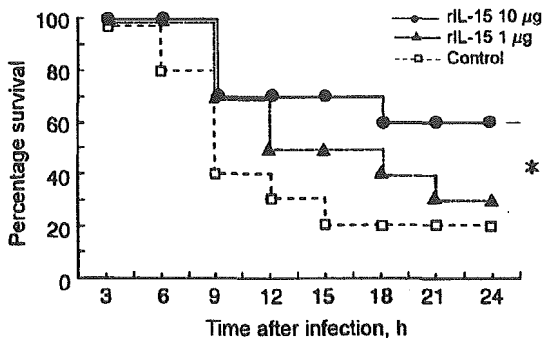

Figure 7. Effects of in vivo administration of recombinant (r) interleukin (IL)–15 on protection against lethal infection with *Escherichia coli*. rIL-15 (1 or 10 μg in 200 μL of PBS) or PBS (200 μL) was intraperitoneally (ip) injected immediately after ip inoculation of 10^9 cfu of *E. coli*, and survival was monitored for 48 h. Each experiment used 7–10 mice/group. Data are typical of 1 of 3 independent experiments. Difference between survival curves of 10 μg of rIL-15 group vs. control group was statistically significant, $^*P < .05$ (log-rank test).

In fact, resistance to apoptosis was coupled to improved survival rate, and peritoneal adherent cells from naive IL-15 Tg mice were resistant to in vitro apoptosis induced by in vitro stimulation with TNF-α, which is mainly responsible for triggering tissue injury and lethal shock. The resistance of cells from IL-15 Tg mice to TNF-α–induced apoptosis was reversed by neutralization of endogenous IL-15. Therefore, we conclude that overexpression of IL-15 prevents apoptosis of host cells and, consequently, improves survival after *E. coli* infection. It remains to be determined whether IL-15/IL-15Rα directly inhibits the signal cascades for apoptosis from TNFR or activates the survival signaling via TRAF-2 recruitment to IL-15Rα.

One of the most widely studied regulators of cell death is the Bcl-2 superfamily [35]. This family has >15 members in humans, mice, and worms. Bcl-2 is an intracellular protein that prevents cell death in a variety of conditions [41, 42]. It protects against cell death by preventing release of cytochrome *c* after mitochondrial damage and plays a critical role in the regulation of cell survival and apoptosis [36]. IL-15 maintains high levels of Bcl-2 at transcriptional levels via activation of transcription factors such as STAT-5 and c-Myb [28]. It can be speculated that overexpression of IL-15 in vivo enhances the expression of Bcl-2 after *E. coli* infection, resulting in maintenance of peritoneal cell levels.

We found that peritoneal adherent cells from IL-15 Tg mice infected with *E. coli* expressed more Bcl-2 proteins than did cells from non-Tg mice. This suggests that overexpression of IL-15 enhances the expression of Bcl-2 and results in maintenance of cell levels. In addition to Bcl-2, other Bcl-2 family members (e.g., Bcl-w, Bcl-xl, Bcl-xs, and A1) are also antiapoptotic proteins for cell survival [43]. Among them, A1 is a rapidly inducible gene that was initially characterized in murine macrophages. We reported elsewhere that IL-15 prevents mouse mast cells from apoptosis through STAT6-mediated Bcl-xL expression [44]. Further investigation is required to determine whether other antiapoptotic proteins are involved in protection against apoptosis by IL-15 overexpression.

A notable finding in the present study was that neutralization of endogenous IL-15 by sIL-15Rα aggravated TNF-α–induced apoptosis in peritoneal adherent cells from normal mice. This suggests that normal macrophages may produce IL-15 in response to TNF-α and protect themselves against apoptosis in an autocrine manner. In a physiologic situation, IL-15 may play an important role in protection of cells against various stimuli for apoptosis. To confirm this possibility, we are now carrying out experiments in IL-15 knockout mice.

In summary, we found that IL-15 Tg mice had resistance to *E. coli*–induced septic shock accompanied by inhibition of TNF-α–induced apoptosis. Treatment with exogenous IL-15 rendered normal mice resistant to lethal shock induced by *E. coli* infection. These findings suggest a possible use of IL-15 in prophylactic and therapeutic approaches for controlling lethal septic shock induced by gram-negative bacteria through prevention of multiple organ failure with apoptosis.

Acknowledgments

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NK T cells stimulated with a ligand for TLR2 at least partly contribute to liver injury caused by *Escherichia coli* infection in mice

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Fas ligand (Fas L) expression was induced on intrahepatic NK1.1$^+$ T cells *in vivo* after an intraperitoneal inoculation of *Escherichia coli*. Liver injury after *E. coli* infection, as assessed by serum GPT level and histological examination, was significantly reduced in Jα281$^{-/-}$ mice lacking NK1.1$^+$ T cells or in *gld/gld* mice bearing mutated Fas L, indicating that NK T cells at least partly contribute to *E. coli*-induced liver injury in a Fas/Fas L-dependent manner. Bacterial numbers in organs and cytokine levels in serum of Jα281$^{-/-}$ mice did not differ from those of Jα281$^{+/+}$ mice following *E. coli* infection. Intrahepatic NK1.1$^+$ T cells, which preferentially expressed Toll-like receptor 2 (TLR2) mRNA, responded *in vitro* to synthetic lipoprotein, a ligand for TLR2, by inducing Fas L expression on their surface. In a manner analogous to *E. coli* infection, lipoprotein and LPS could additively induce Fas L expression on NK1.1$^+$ T cells, leading to liver injury *in vivo* in normal mice but not in *gld/gld* mice. In conclusion, it is suggested that induction of Fas L on NK T cells in response to bacterial components such as lipoproteins plays an important role in pathogenesis of *E. coli*-induced liver injury in mice.

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1 Introduction

The incidence of infection with *Escherichia coli* has increased in recent years among the patients undergoing abdominal surgery. These infections frequently result in liver injury and fatal shock, which are caused by endotoxin/ LPS derived from gram-negative bacteria [1]. Massive TNF-α and IL-1β released from macrophages play a central role in LPS-induced liver injury [2, 3]. IL-12 and IL-18 are also known to play important roles in LPS-induced liver injury [4, 5].

Mouse liver contains unique αβ T cells expressing NK1.1 Ag, a large fraction of which express an invariant TCR encoded by Vα14 and Jα281 gene segments [6, 7] and are specialized to recognize the processed glycolipids from enteric bacteria such as *E. coli* [8]. Intrahepatic NK1.1$^+$ T cells are found to be a target of IL-12 for NK-like effector mechanisms such as IFN-γ production and Fas ligand (Fas L)-mediated cytotoxicity [4, 9–12]. There are several lines of evidence to suggest that NK T cells are involved in the formation of liver damage in animal models [13, 14]. However, it remains to be elucidated whether NK T cells in the liver contribute to liver damage induced by infection with extracellular bacteria such as *E. coli*.

Toll was first identified as a protein controlling dorsoventral pattern formation in the early development of *Drosophila* [15] and was shown to participate in antimicrobial immune responses [16]. Several mammalian Toll homologues have been identified and shown to play important roles in the recognition of various bacterial components. Toll-like receptor (TLR) 2 and TLR4 have been shown to be involved in signaling from lipoproteins (LP) and lipid A, respectively, whereas TLR9 has been shown to play a crucial role in recognition of bacterial DNA (CpG-DNA) [17–19]. TLR3 and TLR5 have been reported to recognize double-stranded RNA (dsRNA) and bacterial flagellin, respectively [20, 21]. TLR2 is

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Abbreviations: Fas L: Fas ligand **TLR:** Toll-like receptor **LP:** Lipoprotein **GPT:** Glutamic-pyruvic transaminase **-/-:** Knockout **mRNA:** Messenger RNA

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expressed not only on phagocytes but also on some subsets of T cells including γδ T cells and NK1.1$^+$ T cells [22, 23]. We have recently reported that TLR2 can at least partly contribute to liver injury caused by infection with an intracellular gram-negative bacteria, *Salmonella choleraesuis* via Fas L induction on liver NK T cells [13, 23].

In the present study, we focused on the roles of NK T cells and TLR2 on their surface in pathogenesis of liver injury induced by *Escherichia coli* infection. We found that induction of Fas L expression on NK T cells by a TLR2 ligand, lipoprotein, plays an important role in liver injury caused by *E. coli* infection.

2 Results

2.1 Contribution of Fas L on NK T cells to liver injury after *E. coli* infection

We have previously reported that Fas L expression was selectively induced on NK T cells in liver 7 days after *Salmonella* infection [23]. To determine whether Fas L expression was induced on NK T cells *in vivo* by *E. coli* infection, we analyzed the expression of Fas L on intrahepatic lymphocytes of C57BL/6 mice 12 h after intraperitoneal inoculation with *E. coli* at a dose of 1.0×10^9 CFU/mouse by flow cytometry. As shown in Fig. 1, the level of Fas L expression on NK T cells was apparently increased after *E. coli* infection, although the relative number of NK T cells in whole liver lymphocytes was decreased (Table 1). On the other hand, the expression level on NK cells or T cells did not significantly increase after infection. Thus, Fas L was preferentially upregulated on NK T cells after *E. coli* infection.

To investigate the involvement of Fas L on liver NK T cells in liver injury following *E. coli* infection, we next compared the serum glutamic-pyruvic transaminase (GPT) levels in Jα281$^{-/-}$ and *gld/gld* mice with non-functional Fas L expression and those in B6 control mice after infection of *E. coli*. The serum GPT levels in Jα281$^{-/-}$ and

Table 1. Proportion of intrahepatic lymphocyte subset[a]

	NKT	NK	T cell
Naive	20.07±2.02	12.41±0.41	32.83±6.28
Infected	11.94±2.54[b]	18.03±5.84[b]	36.34±7.98

[a] Values are given as % (mean ± SD) of five individual experiments.

[b] $p<0.01$ by Student's *t*-test compared with the values for each naive group, respectively.

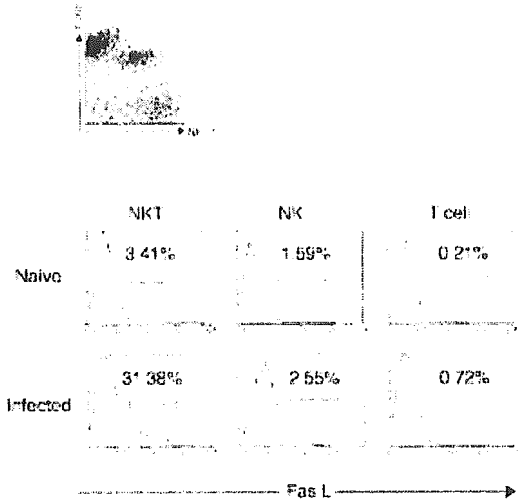

Fig. 1. Expression of Fas L on intrahepatic lymphocytes after *E. coli* infection. Intrahepatic lymphocytes were isolated from the livers of naive and *E. coli*-infected C57BL/6 mice (1.0×10^9 CFU). Intrahepatic lymphocytes were stained with anti-TCRβ, anti-NK1.1 and anti-Fas L mAb and analyzed with a flow cytometer. Data are representative of three separate experiments.

gld/gld mice were significantly lower than those in control B6 mice at 12 h after inoculation with 1.0×10^9 CFU of *E. coli* (Fig. 2, $p<0.05$).

The tissues of liver of *E. coli*-infected Jα281$^{-/-}$, *gld/gld*, and control B6 mice were obtained at 12 h after infection. Histological examination stained with hematoxylin and eosin confirmed that necroinflammatory foci, cell death of hepatocytes characterized by cell shrinkage and chromatin condensation, and lymphocyte infiltration were obviously less in Jα281$^{-/-}$ and *gld/gld* mice than in control mice after infection (Fig. 3A). Apoptotic cells were next identified using the TUNEL technique. After *E. coli* infection, higher numbers of cells undergoing apoptosis could be observed in liver of control mice. The apoptotic cells showed characteristic shrinking, chromatin clumping, and nuclear fragmentation. Most of the events in the liver were located in the cells lining the sinusoids. In contrast, the amount of apoptotic cells remained low in Jα281$^{-/-}$ and *gld/gld* mice (Fig. 3B). These results suggest that the Fas/Fas L system mediated by liver NK T cells could contribute to liver injury following *E. coli* infection.