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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得　　該当なし
2. 実用新案登録　該当なし
3. その他　　該当なし

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（業務項目）

メトホルミンによる腫瘍局所免疫疲弊解除に基づく癌免疫治療に関する研究

業務項目： 副作用管理に関する検討

担当責任者　　和田　淳　　岡山大学大学院医歯薬学総合研究科　准教授

研究要旨
メトホルミンは1990年代に2型糖尿病に対する大規模な臨床試験が行われ、肥満抑制効果、肥満者における大血管障害の抑制、癌発生抑制効果が証明され、欧米では2型糖尿病治療の第1選択薬である。メトホルミンの発売後調査のデータをもとにして消化器症状と乳酸アシドーシスについてその頻度や症状について検討し、健常者や悪性腫瘍患者に対するメトホルミンを用いた研究の用量設定を行った。消化器症状は1500mg/日で漸増法を行うことで回避できると考えられた。またまたメトホルミンの添付文書に記載している禁忌事項はそのまま除外項目とすることにより重篤な副作用である乳酸アシドーシスが回避できる。

A. 研究目的

メトホルミンの合成は 1929 年にさかのぼるがインスリンの登場で糖尿病治療薬としては忘れ去られていた。1950 年代後半にフランスの医師 Jean Sterne が糖尿病薬として再評価を発表して臨床に用いられるようになったが、アメリカでは同じビグアナイド薬であるフェンホルミンによる死亡率の高い乳酸アシドーシスのまえビグアナイド薬は一切使用が中止されていた。一方 1990 年代に 2 型糖尿病に対する大規模な臨床試験が行われ、肥満抑制効果、肥満者における大血管障害の抑制、癌発生抑制効果が証明された。米国でも 1994 年に FDA がメトホルミンを認可し、欧米では 2 型糖尿病治療の第一選択薬となった。

メトホルミンの健常者における免疫機能への影響や、悪性腫瘍におけるメトホルミンの抗腫瘍効果を検討する上で、メトホルミンの副作用に配慮した投与計画を検討した。

B. 研究方法

メトホルミンの発売後調査のデータをもとにして消化器症状と乳酸アシドーシスについてその頻度や症状について検討した。

（倫理面への配慮）

健常者や悪性腫瘍患者においてメトホルミンをより安全に内服していただけるように配慮した。

C. 研究結果

消化器症状

消化器症状は169例中98例(58.0%)に認められ、下痢・悪心、嘔吐、食欲不振などであるが、日常生活がおくれないような重度の症例はなく、97例で日常生活に支障を来さない軽度の症状であった。1500mg/日の症例（120例）では8週まで、2250mg/日の症例では12週ころまで発症例が増加する。従ってメトホルミンの消化器症状は漸増法を用いることによって発現を抑えることができる。

乳酸アシドーシス

2010年５月から2014年9月30日の間に59例の報告があり、35-44歳2例、45-54歳8例、55-64歳12例。65歳-74歳16例で、75歳以上が20例であり、死亡例は10例であった。また250mg/日、500mg/日の低用量でも発症が認められた。慢性の背景因子としては75歳以上(34%)、心血管系疾患(33%)、慢性腎不全・腎機能低下（31%）、飲酒（29%）、慢性肝障害（12%）、脱水（72%）などがあり、急性の背景因子としては腎機能の悪化（50%）、感染症（26%）があった。ほとんどの症例で禁忌

事項を伴っており、禁忌の症例には投与しないことが重要であると考えられる。また脱水・シックデイ、過度のアルコール摂取の患者では禁忌であり、利尿薬やSGLT2阻害薬との併用時には特に脱水に注意する必要がある。

D. 考察

健常者に対するメトホルミン投与は 1500mg/日で２週間を予定しており、消化器症状の出現が予測される。症状によっては減量をするべきだと考えられる。また悪性腫瘍を有する患者の投与では、脱水や食欲不振を来しやすいので500mg/日３日間より開始して、750mg/日４日間投与の後、1500mg/日に増量することが望ましいと考えられた。また症状によっては４週間くらい時間をかけながら増量することが重要であることが考えられる。乳酸アシドーシスはメトホルミンが禁忌の患者で発症しているのでメトホルミンの禁忌事項をそのまま除外基準とすることが肝要である。

E. 結論

対象者へのメトホルミンの投与は1500mg/日としたが、悪性腫瘍患者においては漸増法を行い、場合によってはゆっくりと漸増する配慮が必要である。またメトホルミンの添付文書に記載している禁忌事項はそのまま除外項目とすることが望ましい。

F. 健康危険情報

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H.　知的財産権の出願・登録状況（予定を含む）

1. 特許取得　　該当なし
2. 実用新案登録　該当なし
3. その他　　該当なし

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（総括・業務項目）

メトホルミンによる腫瘍局所免疫疲弊解除に基づく癌免疫治療に関する研究

業務項目： 生物統計学に関する検討

担当責任者　　樋之津　史郎　　岡山大学病院　教授

研究要旨

癌免疫療法における有効性の指標を評価するために、動物モデルで用いられたリンパ球の多機能性評価をヒトに用いる事の妥当性について検討した。

研究結果から得られた末梢血リンパ球の多機能性についての定量的評価は、閾値の設定について検討が必要であるが、評価しようとして妥当である事が示された。今後、予後情報などとの関連を検討することが必要である。

A. 研究目的

癌免疫治療は、低分子化合物などの抗癌剤による治療に比べて有効性の指標を何に設定するか明確ではない。今回の研究では、エンドポイント設定の妥当性について検討する事を目的とした。

B. 研究方法

動物モデルでの研究で得られたリンパ球の多機能性評価を、ヒトに用いる事が妥当かどうか検討した。また、それらの指標が、再発率や生存率と関連しているかを検討した。

（倫理面への配慮）

研究計画書は、健常者および糖尿病患者を対象とする場合、施設内の倫理委員会に計画書を提出し審査の上、承認後に研究を行った。

C. 研究結果

健常者を対象とした研究および2型糖尿病患者を対象とした研究において、メトホルミン内服前後の免疫能機能評価を、末梢血リンパ球の多機能性を定量的に評価することでじっしかのうであることを確認した。

D. 考察

今回得られた定量的評価における閾値の設定と、今後得られる予後情報との関連を考慮して、感度・特異度等を算出する事が可能であることが示唆される。

E. 結論

末梢血リンパ球の多機能性定量的評価は癌免疫治療の評価として有用である事が示唆された。

F. 健康危険情報

総括の項を参照

G. 研究発表

1. 論文発表

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該当なし

H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得　　該当なし
2. 実用新案登録　該当なし
3. その他　　該当なし

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（業務項目）

メトホルミンによる腫瘍局所免疫疲弊解除に基づく癌免疫治療に関する研究

業務項目： 治験プロトコールに関する検討

担当責任者　　平田　泰三　　岡山大学病院　准教授

研究要旨
２型糖尿病治療薬メトホルミンの長期服用により癌の発症率・癌死率が低下する。このメカニズムに関し、我々は動物モデルにおいて、T細胞の免疫疲弊を回避し、さらに疲弊状態からの回帰を起こすことを明らかにした。この知見がヒトでも適用されることを証明し、メトホルミンによる癌患者に対する有効性と安全性の確認、並びに適応拡大を目指した治験を含めた臨床開発推進を行っていく。

A. 研究目的

メトホルミンによる腫瘍局所におけるT細胞の免疫疲弊を回避、さらに疲弊状態からの回帰を起こすことが動物モデルで明らかにされている。この知見がヒトでも適用されることを証明し、メトホルミンによる癌患者に対する有効性と安全性の確認、並びに適応拡大を目指した治験を含めた円滑な臨床開発推進を行っていく。

B. 研究方法

我々は、アカデミア臨床研究機関（Academic Research Organization　以下ARO）として，早期段階の臨床開発，ARO主導の臨床試験においてプロトコールの作成，試験実施施設の選定，ICH-GCP準拠の臨床試験及び治験の実施、データのマネジメント，得られたデータの解析等を行い、臨床開発においてそのコンセプトの妥当性を傍証することでproof of conceptを構築し、製薬企業との橋渡しを行い検証試験まで繋いでいき、最終的には薬事承認取得を目指す臨床開発を行っていく。

（倫理面への配慮）

当該研究で実施する臨床研究あるいは治験について、研究者等はヘルシンキ宣言に基づく倫理原則及びGCP省令、各府庁の定める省令及び指針、個人情報保護法等を遵守する。

C. 研究結果

ヒトにおけるメトホルミン非服用時の癌性胸水・腹水・末梢血及びTILの疲弊の程度を明らかにする臨床研究を作成し、併せてTreg，MDSCの表面分子とサイトカイン産生について検討している。今後は、担癌患者に対するメトホルミン投与の介入研究を行い、癌性胸水・腹水・末梢血とCD8TILの疲弊解除及びTreg，MDSCについて比較検討する予定である。

D. 考察

担癌患者において、メトホルミンの投与・非投与時の癌性胸水・腹水・末梢血及びTILの疲弊の程度を明らかにすることにより、Proof of Concept（POC)を確立しつつある。
得られたデータをもとに、癌患者に対する有効性と安全性について評価可能な治験デザイン設計を今後、行っていく。

E. 結論

担癌患者に対する有効性と安全性を確認並びに適応拡大を目指した治験を含めた円滑な臨床開発推進を行っていく。

F. 健康危険情報

総括の頁を参照。

G. 研究発表

1. 論文発表
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EGFR-TKI 耐性の EGFR 変異陽性 NSCLC を対象とした tivantinib (ARQ 197)とエルロチニブ併用の第 2 試験, 日本肺癌学会. 2014/11/1

H. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得　　該当なし
2. 実用新案登録　該当なし
3. その他　　該当なし

III. 学会等発表実績

様式第１９

学　会　等　発　表　実　績

委託業務題目「メトホルミンによる腫瘍局所免疫疲弊解除に基づく癌免疫治療研究」
機関名　国立大学法人岡山大学

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Metformin-induced reversion of exhausted CD8T cells in tumor microenvironment（コア・シンポジウム）	Udono H, Nishida M, Eikawa S.	73rd Annual Meeting of the Japanese Cancer Association	平成26年9月27日	国内
メトホルミンによる腫瘍局所の免疫疲弊解除（シンポジウム）	鵜殿平一郎、榮川伸吾	第87回日本生化学会大会	平成26年10月17日	国内
エネルギーセンサーを介したストレス応答と腫瘍免疫の関わり（シンポジウム）	鵜殿平一郎	第9回臨床ストレス応答学会	平成26年11月2日	国内
メトホルミンによる腫瘍局所の免疫疲弊解除（教育講演）	鵜殿平一郎	第12回日本免疫治療学研究会学術集会	平成27年2月28日	国内
Metformin-induced tumor infiltrating CD8 T cells with effector memory phenotype and multi-functional reversion.	Eikawa S, Negawa M, Kunisada Y, Uehara T, Ichiyanagi T, Ymazaki C. Udono H.	The 73rd Annual Meeting of the Japanese Cancer Association	平成26年9月26日	国内
腫瘍浸潤CD8T細胞の免疫疲弊解除における活性化AMPKの役割	榮川伸吾，根川真実，國定勇希，上原健敬，一柳朋子，山﨑千尋，鵜殿平一郎	日本がん免疫学会	平成26年11月2日	国内
Activated AMPK induced tumor infiltrating CD8 T-cells with effector memory phenotype and functional reversion	Eikawa S, Negawa M, Kunisada Y, Uehara T, Ichiyanagi T, Yamazaki C, Udono H	The 73rd Annual Meeting of the Japanese Cancer Association	平成26年9月26日	国内
2型糖尿病薬メトホルミンの腫瘍局所におけるCD8 T細胞疲弊解除	榮川伸吾	第9回臨床ストレス応答学会	平成26年11月1日	国内
Immunomonitoring multifunctionality of exhausted CD8 T-cells in cancer patients	Negawa M, Eikawa S, Uehara T, Kunisada Y, Ohue Y, Kurose K, Isobe M, Uenaka A, Kakimi K, Wada H, Oka M, Nakayama E, Udono H.	The 73rd Annual Meeting of the Japanese Cancer Association	平成26年9月26日	国内
Metformin induced tumor infiltrating CD8 T effector memory cells with multiple cytokine producing ability	Eikawa S, Negawa M, Kunisada Y, Uehara T, Nishida M, Ichiyanagi T, Yamazaki C, Udono H.	日本免疫学会総会・学術集会	平成26年12月10日	国内
医療時代における外科医ー研究心という剪刀を携えるー（シンポジウム）	豊岡伸一	日本外科学会	平成26年4月	国内
悪性胸膜中皮腫におけるマイクロ RNA 異常（シンポジウム）	豊岡伸一	日本産業衛生学会	平成26年5月	国内
肺癌新規遺伝子異常の発見ー最適化医療時代における外科医の役割を考えるー（要望演題）	豊岡伸一	日本呼吸器外科学会	平成26年5月	国内
呼吸器外科領域における周術期管理について（口演）	豊岡伸一	臨床呼吸生理研究会	平成26年6月	国内
肺癌治療の発展における外科医の役割（口演）	豊岡伸一	日本癌学会	平成26年9月	国内

Virus-guided fluorescence imaging of intraperitoneal free gastric cancer cells as a potential clinical biomarker. (ポスター)	Watanabe M, Kagawa S, Ishida M, Hori N, Kikuchi S, Kuroda S, Kishimoto H, Nishizaki M, Tazawa H, Fujiwara T	Annual Meeting of American Association for Cancer Research 2014	平成26年4月	国外
Genetic/epigenetic変異を基盤とした大腸癌個別化治療の構築. (口演：シンポジウム))	永坂岳司、母里淑子、稲田 涼、岸本浩行、近藤喜太、浅野博昭、佃 和憲、西崎正彦、香川俊輔、藤原俊義	第69回日本消化器外科学会総会	平成26年7月	国内
消化器がんにおける遺伝子改変ウイルスを用いたセンチネルリンパ節転移アブレーション. (口演：シンポジウム)	菊地寛次、岸本浩行、田澤 大、黒田新士、西崎正彦、香川俊輔、浦田泰生、Robert Hoffman、藤原俊義	第16回SNNS研究会学術集会	平成26年9月	国内
A feasibility study To visualize intraperitoneal disseminated gastric cancer by fluorescence-emitting virus, TelomeScan. (口演)	Watanabe M, Kagawa S, Ishida M, Hashimoto Y, Hori N, Kikuchi S, Kuroda S, Kishimoto H, Nishizaki M, Tazawa H, Urata Y, Fujiwara T	第73回日本癌学会学術総会	平成26年9月	国内
ACAM (adipocyte adhesion molecule) /CLMPの一次繊毛機能を介した脂肪細胞分化と肥満症における意義(口演)	村上和敏、和田 淳、佐藤美和、江口 潤、布上朋和、片山晶博、中司敦子、小川大輔、四方賢一、槇野博史	第57回日本糖尿病学会総会	平成26年5月24日	国内
メタボリックシンドロームにおけるphosphatidylethanolamine N-methyltransferase (PEMT) の意義 (口演)	中司敦子、和田 淳、村上和敏、勅使川原早苗、片山晶博、渡邊真由、樋口千草、天田雅文、布上朋和、江口 潤、小川大輔、槇野博史	第57回日本糖尿病学会総会	平成26年5月24日	国内
肥満により脂肪組織に誘導される膜蛋白Gpnmbの脂肪肝炎抑制効果 (口演)	片山晶博、和田 淳、中司敦子、江口 潤、村上和敏、勅使川原早苗、樋口千草、布上朋和、天田雅文、肥田和之、槇野博史	第57回日本糖尿病学会総会	平成26年5月24日	国内
脂肪肝炎におけるPemtの意義 (口演)	中司敦子、松山 誠、村上和敏、勅使川原早苗、江口 潤、小川大輔、高木章乃夫、福島正樹、山本和秀、槇野博史、和田淳	第１回肝臓と糖尿病・代謝研究会　伊藤謝恩ホール、東京	平成26年7月4日	国内
臨床研究・治験におけるＩＣＴ活用の現状と今後　臨床研究中核病院の取り組み (口頭)	平田泰三	大学病院情報マネジメント部門連絡会議	平成27年2月	国内
進行肺癌における少量補液法を用いたシスプラチン ベース化学療法の忍容性試験 (口頭)	平田泰三、久本晃子、市原英基、堀田勝幸、瀧川奈義夫、田端雅弘、谷本光音、木浦勝行	第110回日本内科学会講演会	平成26年4月	国内
肺に孤立性腫瘤を形成した非喫煙者のランゲルハンス細胞組織球症の一例 (口頭)	田村朋季、後藤田裕子、中村香葉、萱谷紘枝、平田泰三、佐藤晃子、田端雅弘、木浦勝行、谷本光音	11回日本臨床腫瘍学会学術集会	平成26年8月	国内

PhaseII study of erlotinib plus tivantinib in patients with EGFR mutation positive NSCLC who failed in immediately previous EGFR TKI therapy , poster session	Tomonori Hirashima, Koichi Azuma, Nobuyuki Yamamoto, Toshiaki Takahashi, Makoto Nishio, Taizo Hirata, Kaoru Kubota, Kazuo Kasahara, Toyoaki Hida, Hiroshige Yoshioka, Kohei Suzuki, Shiro Akinaga, Kazuto Nishio, Tetsuya Mitsudomi, Kazuhiko Nakagawa	American society of clinical omcology annual meeting	平成26年6月	国外
EGFR-TKI耐性のEGFR変異陽性NSCLCを対象としたtivantinib (ARQ 197)とエルロチニブ併用の第2試験	金田裕靖、東 公一、平島智徳、山本信之、高橋利明、西尾誠人、平田泰三、久保田 馨、笠原寿郎、樋田豊明、吉岡弘鎮、鈴木康平、秋永士朗、西尾和人、光冨徹哉、中川和彦	日本肺癌学会	平成26年11月	国内

2．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Immune-mediated anti-tumor effect by type 2 diabetes drug, metformin.	Eikawa S, Nishida M, Mizukami S, Yamazaki C, Nakayama E and Udono H.	Proc. Natl. Acad. Sci. USA 10;112(6):1809-14, 2015. doi: 10.1073/pnas.1417636112.	平成27年2月10日	国外
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Kissing-stents technique after living-donor lobar lung transplantation	Seiichiro Sugimoto, Takahiro Oto, Shinichi Toyooka, Shinichiro Miyoshi	European Journal of Cardio-Thoracic Surgery	平成26年8月21日	国外
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Hsp90 inhibitor NVP-AUY922 enhances the radiation sensitivity of lung cancer cell lines with acquired resistance to EGFR-tyrosine kinase inhibitors	Shinsuke Hashida, Hiromasa Yamamoto, Kazuhiko Shien, Tomoaki Ohtsuka, Ken Suzawa, Yuho Maki, Masashi Furukawa, Junichi Soh, Hiroaki Asano, Kazunori Tsukuda, Shinichiro Miyoshi, Susumu Kanazawa, Shinichi Toyooka	Oncology Reports	平成27年1月20日	国外
Lower lobe origin is a poor prognostic factor in locally advanced non-small-cell lung cancer patients treated with induction chemoradiotherapy	Kazuhiko Shien, Shinichi Toyooka, Junichi Soh, Katsuyuki Hotta, Kuniaki Katsui, Takahiro Oto, Susumu Kanazawa, Katsuyuki Kiura, Hiroshi Date, Shinichiro Miyoshi	Molecular and Clinical Oncology	平成27年2月11日	国外
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（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。
（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

Ⅳ．研究成果の刊行物・別刷

Immune-mediated antitumor effect by type 2 diabetes drug, metformin

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Metformin, a prescribed drug for type 2 diabetes, has been reported to have anti-cancer effects; however, the underlying mechanism is poorly understood. Here we show that this mechanism may be immune-mediated. Metformin enabled normal but not T-cell–deficient SCID mice to reject solid tumors. In addition, it increased the number of $CD8^+$ tumor-infiltrating lymphocytes (TILs) and protected them from apoptosis and exhaustion characterized by decreased production of IL-2, TNFα, and IFNγ. $CD8^+$ TILs capable of producing multiple cytokines were mainly $PD\text{-}1^-Tim\text{-}3^+$, an effector memory subset responsible for tumor rejection. Combined use of metformin and cancer vaccine improved $CD8^+$ TIL multifunctionality. The adoptive transfer of antigen-specific $CD8^+$ T cells treated with metformin concentrations as low as 10 μM showed efficient migration into tumors while maintaining multifunctionality in a manner sensitive to the AMP-activated protein kinase (AMPK) inhibitor compound C. Therefore, a direct effect of metformin on $CD8^+$ T cells is critical for protection against the inevitable functional exhaustion in the tumor microenvironment.

immune exhaustion | CD8T cells | antitumor immunity | tumor microenvironment | multifunctionality

In chronic infectious diseases and cancer, $CD8^+$ T cells specific for viral and/or tumor antigens undergo repeated TCR stimulation because of persistent pathogens or cancer cells and gradually lose their ability to secrete IL-2, TNFα, and IFNγ, eventually undergoing apoptotic elimination in a process known as immune exhaustion (1). This worsening immune function is accompanied by phenotypic changes in $CD8^+$ T cells, including the expression of exhaustion markers such as PD-1 and Tim-3 (2). Antitumor immunity is enhanced in mice deficient in PD-1 or its ligands PDL-1 and PDL-2 (2-4). Galectin 9, a Tim-3 ligand, is secreted by many tumor cells as well as by FoxP3-expressing regulatory T-cell (Treg) and inhibits Tim-3–expressing Th1 cells (5). An anti–Tim-3 antibody that blocks the galectin 9–Tim-3 pathway was found to accelerate antitumor immunity (6). Furthermore, the administration of blocking antibodies against both PD-1 and Tim-3 induced a more profound tumor rejection in comparison with that achieved with either antibody alone (7). The management of functional T-cell exhaustion within tumor tissues is currently an extensive focus in tumor immunotherapy (8, 9), together with efforts to neutralize immune-inhibitory Treg and myeloid-derived suppressor cell (MDSC).

Metformin (dimethylbiguanide) has been widely prescribed for type 2 diabetes. Its unique pharmacological features include its antihyperglycemic efficacy, which counters insulin resistance (10, 11). Early metformin use increases the survival of patients with obesity-involved type 2 diabetes and/or cardiovascular disease (12). In addition, recent reports have described the unexpected anticancer effects of metformin in patients with type 2 diabetes (13). Insulin-based diabetes treatment is associated with an increased cancer risk (14–17), whereas metformin use has been shown to decrease the frequency of specific cancers (18–21). Two independent metaanalyses of epidemiological studies concluded that compared with other treatments, metformin is associated with a 30–40% reduction in the incidence of cancer among patients with type 2 diabetes, indicating the need to investigate the anticancer mechanisms of metformin and conduct long-term randomized controlled trials (RCTs) (22, 23).

In the HER-2/*neu* transgenic mouse breast cancer model, metformin treatment decreased the tumor burden and was associated with an increased life span (24). Combined use of metformin with chemotherapeutic agents such as cisplatin has also yielded clinical benefits (25, 26). Regarding the anticancer mechanism, metformin appears to preferentially kill cancer-initiating/stem cells from glioblastoma (27), breast (28) and ovarian cancers (29) via AMP-activated protein kinase (AMPK) activation.

In contrast to the inhibitory action of metformin on tumor cells, here we demonstrate the direct effects of metformin on $CD8^+$ T cells, which eventually results in tumor growth inhibition. Metformin protects $CD8^+$ tumor-infiltrating lymphocytes (TILs) from apoptosis, and the multifunctionality of exhausted $PD\text{-}1^-Tim\text{-}3^+CD8^+$ TILs is restored via a shift from a central memory (TCM) to an effector memory T-cell (TEM) phenotype. This metformin-induced antitumor mechanism is therefore linked to marked changes in the characteristics of $CD8^+$ TILs within the tumor microenvironment.

Results

Metformin-Induced Tumor Rejection Depends on $CD8^+$T Cells. As metformin has been reported to decrease the rate of cancer incidence in type 2 diabetic patients, we at first examined whether

Significance

The multifunctional ability of CTLs is downregulated by interaction between immune-checkpoint molecules expressed on CTLs and their ligands expressed on cancer cells, referred to as immune exhaustion. The antibody-mediated, immune-checkpoint blockade turned out to a promising method for immunotherapy against advanced melanoma. Metformin, a drug prescribed for patients with type 2 diabetes, has been recognized to have anti-cancer effect. We found that $CD8^+$ tumor infiltrating lymphocytes (TILs) is a target of metformin. $CD8^+$ TILs inevitably undergo immune exhaustion, characterized by diminished production of multiple cytokines such as IL-2, TNFα, and IFNγ, followed by elimination with apoptosis. Metformin is able to counter the state. Along with conventional therapy, treatment of cancer patients with metformin may have a great advantage for cancer therapy.

Author contributions: H.U. designed research; S.E. and M.N. performed research; S.M., C.Y., E.N., and H.U. analyzed data; and H.U. wrote the paper.

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the drug could protect mice from methylchoranthrene-induced skin carcinogenesis. BALB/c mice were injected with 200 μg of methylchoranthrene on the right back and given 5 mg/mL metformin dissolved in the drinking water throughout the experiment. Significant inhibition of tumor development was observed in metformin-treated nondiabetic mice (Fig. S1*A*). We next attempted to determine whether metformin would be effective against an established solid tumor. Mice were intradermally injected with X-ray-induced RLmale1 leukemia cells and were provided oral metformin beginning on day 7. The tumors were gradually and completely rejected with no reappearance after metformin withdrawal. A rechallenge with more than twice the original number of the same tumor cells did not yield mass formation (Fig. 1*A*, *Left*), suggesting the generation of an immunologic memory response. Moreover, the antitumor effect was completely abrogated in SCID mice (Fig. 1*A*, *Right*), clearly demonstrating the necessity of T and/or B cells. Cytotoxic T lymphocytes (CTLs) specific for the tumor antigen peptide pRL1a (30) were generated in mice that rejected the tumor (Fig. S1*B*). Growth inhibition was observed with a metformin dose as low as 0.2 mg/mL (Fig. S1*C*). Of note, a previous report identified the achievement of plasma metformin concentrations of 0.45 and 1.7 μg/mL using 1 and 5 mg/mL of metformin, respectively, in drinking water (31); these plasma concentrations are similar to those in patients with diabetes treated using metformin (0.5–2 μg/mL). Administration of metformin beginning on day 0, the time point of tumor inoculation, resulted in more effective rejection than on day 7. Beginning treatment on day 10 and 13 was also effective, although the effect was less than on day 0 (Fig. S1*D*). Finally, as expected, $CD8^+$ but not $CD4^+$ T cells were proven to be responsible for the antitumor effect, because their depletion by mAb completely abrogated the response (Fig. 1*B*). Complete rejection by metformin was also observed with Renca (renal cell carcinoma), although partial but significant growth inhibition was observed with other tumors, 3LL (non small cell lung carcinoma), Colon 26 (intestinal carcinoma), and 4T1 (breast cancer) (Fig. S1 *E–H*).

Metformin Prevents Apoptosis of $CD8^+$TILs, Irrespective of Expression of PD-1 and Tim-3. Injection of a vaccine consisting of antigen (Ag) and adjuvant primes and generates specific T-cell immunity, mainly in draining lymph nodes near the injection site. However, we did not inject tumor antigens with any kind of adjuvant in Fig. 1. Therefore, it is possible that a unique process occurs at the tumor site and leads to antitumor immunity. Based on this notion, we focused on TILs throughout the experiment to clarify the associated mechanism. We found that total numbers of TILs dramatically increased when metformin administration was started on day 7, and that both $CD8^+$ and $CD4^+$ T cells were involved in the increment (Fig. 1 *C–E*). In particular, the number of $CD8^+$ TILs increased nearly fourfold. We considered the possibility that metformin may suppress expression of the immune exhaustion markers PD-1 and Tim-3 on $CD8^+$ TILs, thus avoiding immune exhaustion. Therefore, we investigated the expression of these markers on $CD8^+$ TILs derived from individual tumor-bearing mice (Fig. S1*B*). The number of PD-1^-Tim-3^- $CD8^+$ TILs decreased from day 7–10, irrespective of metformin use (Fig. S2*B*). The PD-1^-Tim-3^+CD8^+ TIL population increased progressively, whereas PD-1^+Tim-3^- and PD-1^+Tim-3^+CD8^+ TILs remained stable. Metformin did not affect any subset populations (Fig. S2 *B–E*). However, we surprisingly found that a significant proportion of $CD8^+$TILs underwent apoptosis, detected by

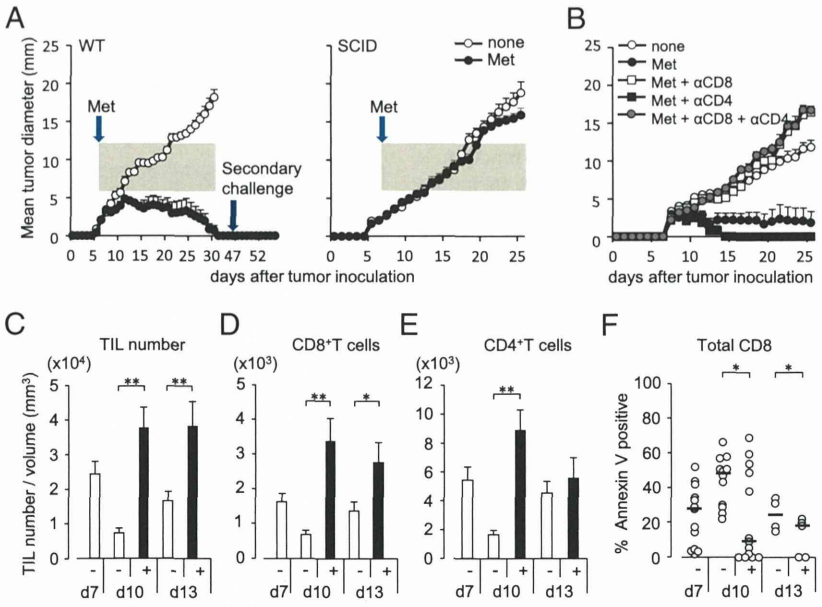


Fig. 1. Metformin suppressed tumor growth in vivo, depending on $CD8^+$ T cells. (*A*) On day 0, BALB/c WT or SCID mice were intradermally inoculated with 2×10^5 RLmale1 cells on the right back. The mice received 5 mg/mL metformin (Met) or not (none) dissolved in the drinking water. The duration of Met administration is indicated by the shaded rectangle. The mean diameter of each tumor was measured every day and the data are plotted with SE. On day 45, Met-treated WT mice, all of which had rejected the tumor, were rechallenged with 5×10^5 RLmale1 cells. $n = 6$ in each group. The results are representative of two independent experiments. (*B*) Mice inoculated with RLmale1 were treated with metformin (Met) or not (none), starting on day 7 and i.v. injected with anti-CD8 mAb and/or anti-CD4 mAb on the same day. Average tumor diameters are plotted with SE. $n = 5$ in each group. (*C–E*) Mice inoculated with RLmale1 cells were treated with Met (+) or not (–) from day 7. On day 7, 10 and 13, the tumor mass was isolated and TILs were recovered. The numbers of TILs per tumor volume (mm^3) were calculated. The numbers of TIL (*C*), $CD8^+$ (*D*), or $CD4^+$ (*E*) per tumor volume are depicted. Also, the populations of $CD8^+$TILs stained with Annexin V were plotted (*F*). All data were with SD ($n = 14$ on days 7 and 10, $n = 5$ on day 13). The horizontal bars indicate median values, and *P* values obtained by two-tailed Student's *t* test are shown as $*P < 0.05$, $**P < 0.01$ $n = 5$–14 in each group. Each symbol represents an individual mouse. The results depicted are a summary of three independent experiments.

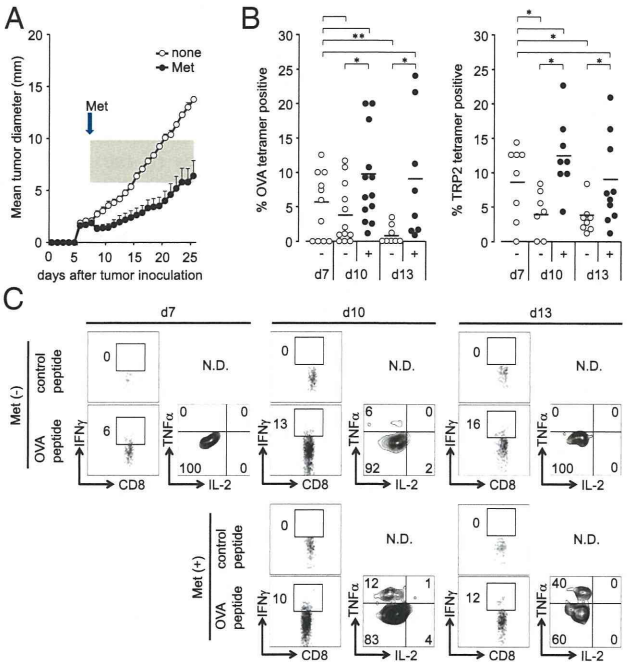


Fig. 2. Metformin improves the multifunctionality of antigen-specific CD8$^+$ TILs in vivo. (*A*) Mice inoculated with 2×10^5 MO5 cells were treated with or without metformin from day 7, as indicated by the shadowed rectangle, and tumor growth was monitored. The results are representative of two independent experiments. $n = 5$ per group. (*B*) On days 7, 10, and 13, TILs were recovered from tumor masses and examined for K^b-$OVA_{257-264}$ and K^b-$TRP2_{180-188}$ tetramer binding ($n = 7$–13). (*C*) TILs recovered on days 7, 10, and 13 from five mice per group [with (+) or without (–) metformin] were pooled and stimulated with DC2.4 cells that had been prepulsed with $OVA_{257-265}$ peptide (10^{-6} M) for 8 h; TIL cytokine-producing ability was later examined.

Annexin V (Fig. 1*F* and Fig. S3*A*), and that metformin suppressed apoptosis induction in all subsets, including PD-1$^-$Tim-3$^+$CD8$^+$TILs (Fig. S3 *B*–*E*). Of note, the physiologically essential apoptotic process of CD4$^+$CD8$^+$ thymocytes, which depends on a mitochondrial pathway (32), was not down-regulated by metformin (Fig. S4), suggesting that an apoptotic mechanism unique to the tumor microenvironment is metformin-sensitive.

We next examined the metformin effects in another tumor system. MO5 is a subclone of B16 melanoma cells expressing ovalbumin (OVA) (33). Metformin administration induced significant antitumor activity (Fig. 2*A*). OVA- and TRP2-specific CD8$^+$ TILs were identified by specific tetramers. Both TIL populations in untreated mice decreased gradually from day 7–13; in contrast, metformin administration maintained or increased these populations (Fig. 2*B*). CD8$^+$ TILs again underwent apoptosis, which was suppressed by metformin administration (Fig. S5 *A* and *B*). The Annexin V-positive populations among OVA tetramer-positive and -negative (includes TRP-2–positive population) CD8$^+$ TILs were near 80% at day 10; however, metformin suppressed this rate to <20–40% (Fig. S5 *C* and *D*). These results are consistent with those observed in the RLmale1 model. Next, to examine the functional state of antigen-specific TILs, magnet-purified CD8$^+$ TILs isolated from tumor tissues were incubated with DC-like DC2.4 cells that had been pulsed with an epitope peptide ($OVA_{257-264}$); TILs were later examined for their cytokine production capacity. Only IFNγ-producing cells or very small populations producing both IFNγ and TNFα or IL-2 could be identified in untreated mice, whereas a marked increase in the population producing both IFNγ and TNFα was observed with metformin (Fig. 2*C*).

Influence of Metformin on the TCM/TEM Ratio of CD8$^+$TILs. CD8$^+$ TILs in the context of memory T cells are poorly understood. Elegant studies with an acute viral infection model have proposed classification of memory T cells into central memory (TCM; CD44$^+$, CD62L^{high}) and effector memory (TEM; CD44$^+$, CD62L^{low}) (34, 35). TCM were shown to mediate viral-specific recall responses. Based on this model, we investigated TCM and TEM CD8$^+$ TILs. Without metformin, the staining of CD8$^+$ TILs from an RLmale1 tumor using antibodies against CD62L and CD44 revealed that proportions of TCM and TEM were nearly equal on day 7 and 10 but shifted to TCM dominance on day 13. In contrast, metformin maintained TEM dominance from day 10 to day 13 (Fig. 3*A*). Further dissection of the TIL compartment based on CD62L and KLRG1 expression revealed that short-lived effector T cells (TE; CD62L^{low}KLRG1^{high}) were visible on day 7 but gradually decreased by day 13. In contrast, metformin yielded increases in both TEM and TE populations on day 13 (Fig. 3*B*), coinciding with tumor regression (Fig. 1*A*). In the MO5 model, metformin again caused TEM dominant over TCM (Fig. 3 *C* and *D*). At this stage, we concluded that TEM and/or TE are more responsible than TCM for tumor rejection.

Metformin Induced Multifunctional CD8$^+$ TEM Expressing the Exhaustion Marker Tim-3. We next investigated the capacity for triple cytokine (IL-2, TNFα, IFNγ) production or the multifunctionality of CD8$^+$ TILs in the context of TCM/TEM classification. CD8$^+$ TILs recovered from RLmale1 tumor masses were stimulated with PMA/ionomycin for 6 h in vitro and monitored for cytokine production. Without metformin, the cytokine-producing cells on day 10 were mainly identified as TCM (Fig. 4*A*). In contrast, with metformin, triple cytokine-producing cells appeared in correlation with the increased population of TEM (Fig. 4*A*). The populations with various cytokine producing patterns in the presence and absence of metformin are summarized in Fig. 4*B*. Metformin markedly changed the multifunctionality of CD8$^+$ TILs. Taking these results together, we concluded that metformin-induced TEM capable of producing

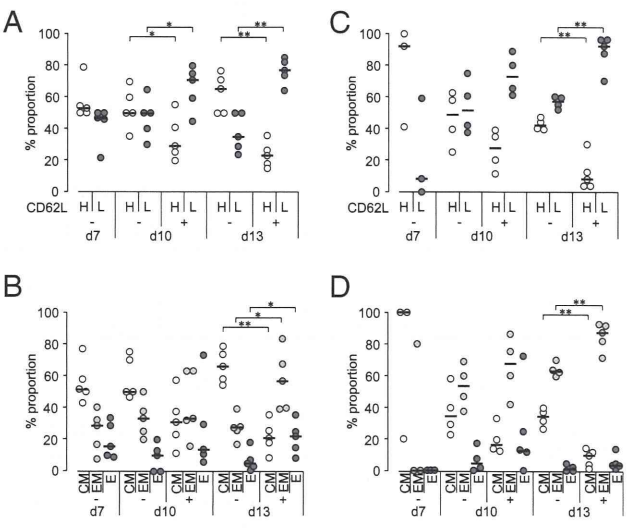
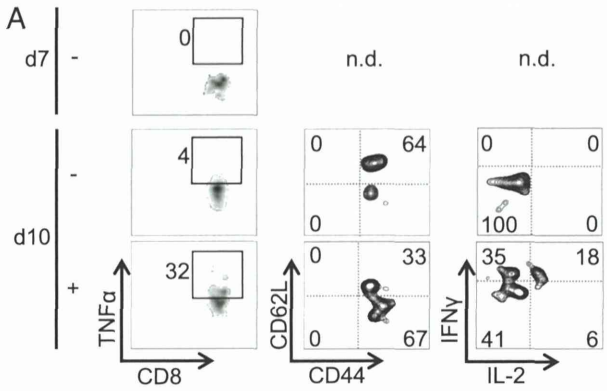


Fig. 3. Influence of metformin on the TCM/TEM ratio of CD8$^+$ TILs. TILs were isolated on days 7, 10, and 13 from mice inoculated with RLmale1 (*A* and *B*, $n = 5$) or MO5 (*C* and *D*, $n = 3$–5) with (+) or without (–) metformin, and analyzed for CD8 and memory markers including CD44, CD62L, KLRG1. The proportion (%) of CD62L^{high} (H) and CD62L^{low} (L) among CD44$^+$ cells in RLmale1 and MO5 models are shown in *A* and *C*, respectively. The proportion (%) of CD62L^{high}, KLRG1^{low} (central memory; CM) and CD62L^{low}, KLRG1 ^{low} (effector memory; EM) and CD62L^{low}, KLRG1 ^{high} (effector; E) in RLmale1 and MO5 are shown in *B* and *D*, respectively. $^*P < 0.05$, $^{**}P < 0.01$.

multiple (triple and double) cytokines are most important for tumor rejection. We next classified $CD8^+$ TILs on the basis of their expression of PD-1 and Tim-3, followed by intracellular cytokine staining. We found that $CD8^+$ TILs with triple cytokine-producing abilities belonged exclusively to the PD-1^-Tim-3^+ subset, which was the supposedly exhausted population in the RLmale1 tumor model (Fig. S6). We further confirmed this notion using adoptive transfer experiments. MO5-inoculated mice were adoptively transferred with OT-I $CD8^+$ T cells. The transferred T cells had been previously shown to undergo vigorous division and were thus cross-primed in vivo via the adjuvant-free administration of a fusion protein comprising OVA and *Mycobacterium* heat shock protein 70 (OVA-mHSP70) as a vaccine (36, 37). OVA-mHSP70 injection significantly enhanced the migration of the transferred CD45.1^+OT-I $CD8^+$ T cells into the tumor tissues; however, the cytokine-producing abilities of these cells were poor (Fig. 5*A*). In contrast, injection of the fusion protein together with oral metformin administration apparently improved the multifunctionality of the migrated T cells, which were classified as the Tim-3^+ population (Fig. 5*A*).



B

cytokine	Met (-)	Met (+)
IL-2^+ TNFα^+ IFNγ^+	-	4.6-7.2% (169-264)
IL-2^+ TNFα^+ IFNγ^-	-	1.3-1.9% (48-69)
IL-2^+ TNFα^- IFNγ^+	-	5.3-7.2% (194-264)
IL-2^- TNFα^+ IFNγ^+	-	7.2-11.2% (264-411)
IL-2^+ TNFα^- IFNγ^-	2.0% (15)	1.7% (62)
IL-2^- TNFα^+ IFNγ^-	4.0% (30)	13.1% (480)
IL-2^- TNFα^- IFNγ^+	9.0% (67)	36.0% (1320)

Fig. 4. Metformin-induced $CD8^+$TILs with multifunctionality are TEM rather than TCM. (*A*) TILs were isolated on the indicated days from five mice per group inoculated with 2×10^5 RLmale1. Met treatment was started (+) or not (–) from day 7. TILs were then pooled on indicated days and stimulated with PMA/ionomycin for 6 h, stained for surface molecules including CD8, CD44, CD62L, followed by intracellular staining for IL-2, TNFα, and IFNγ. $CD8^+$TILs producing TNFα were further analyzed for expression of CD62L and CD44 to identify TCM and TEM. Also, to investigate multifunctionality, cytokine-producing $CD8^+$TILs were further examined for production of IFNγ and IL-2. (*B*) Summary of the populations of cytokine producing $CD8^+$TILs on day 10 is shown. Gated populations for $CD8^+$IFNγ^+, $CD8^+$ TNFα^+, or $CD8^+$IL-2^+ were further analyzed for their production of TNFα and IL-2, IFNγ and IL-2, or IFNγ and TNFα. The gating strategy gives rise to some ranges for % populations of double and triple cytokine producing TILs. The numbers within parenthesis indicate numbers of corresponding $CD8^+$TILs per tumor volume (mm^3).

Metformin-Treated Antigen-Specific Naïve CD8 T Cells Migrate into Tumors and Exert Antitumor Immunity Following Adoptive Transfer. It is unknown whether plasma metformin concentrations as low as 10 μM (1.6 μg/mL) would directly influence the fate of T cells. To address this important question, we incubated $CD8^+$ T cells isolated from naïve OT-I mice with 10 μM metformin for 6 h in the presence or absence of different doses of the AMPK inhibitor compound C (38) as indicated (Fig. 5*B*). After extensive washing, the cells were transferred into MO5-bearing mice. Two days later, splenic T cells and TILs were recovered and investigated for the presence and multifunctionality of donor-derived $CD8^+$ T cells. Metformin-treated $CD8^+$ TILs comprised up to 9.9% of all $CD8^+$ T cells and were identified as triple cytokine-producing cells (Fig. 5*B*). However, compound C treatment abrogated the migration, although donor $CD8^+$ T cells were present in the spleens of all groups (Fig. 5*B*). Accordingly, tumor growth inhibition was apparent in the metformin-treated group, although this effect was blocked by compound C (Fig. 5*C*). The weak but significant metformin-mediated increase in the phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC) and the abrogation of this effect by compound C were observed by Western blot analysis (Fig. 5*D*). The results led us to conclude that the direct action of metformin on $CD8^+$ T cells, at least partly, reduced their exhaustion within the tumor microenvironment in a manner sensitive to the AMPK inhibitor compound C.

AMPK Phosphorylation, Enhanced Bat3 Expression, and Caspase-3 Inhibition Mediated by Metformin. Finally, we examined the expression of $CD8^+$ TIL molecules that may possibly be influenced by metformin administration. After $CD8^+$ TIL purification on day 10, cell lysates were immediately prepared for candidate molecule detection via Western blot analysis and for caspase-3 activity measurement using a fluorescent substrate. The levels of phosphorylated AMPKα and β were increased; a twofold increase in Bat3 expression was also observed, whereas Bcl2 and Bax expression were unaltered (Fig. S7*A*). As expected, caspase-3 activity was prominent without metformin but was completely abrogated in $CD8^+$ TILs from metformin-treated mice (Fig. S7*B*), which offers a plausible explanation for apoptosis inhibition. To further examine the apoptotic cell populations, we evaluated the expression of active caspase-3 in TCM, TEM, and TE. Without metformin, TCM, TEM, and TE all expressed active caspase-3 whereas with metformin, primarily TCM expressed this activated enzyme (Fig. S7*C*). These results may explain the dominance of TCM over TEM in the absence of metformin and the dominance of TEM and TE in the presence of metformin. pS6, a downstream target of mTOR, was positive in TCM, TEM, and TE without metformin but negative with metformin (Fig. S7*D*), indicating that metformin inhibits mTOR, possibly via AMPK activation.

Discussion

In this report, we showed that established solid tumors are regressed by oral administration of metformin, and that $CD8^+$T cells mediate this effect. The number of FoxP3 expressing $CD4^+$ regulatory T cells (Treg) has been implicated as a critical component in suppressing tumor immunity (39). However, their numbers were not decreased, rather, transiently increased by metformin administration in RLmale 1 tumor model (Fig. S8). Upon tumor rejection, the treated mice became resistant to rechallenge with the same tumor, providing proof of memory T-cell generation. Because no protective effect was observed in SCID mice, the direct killing of tumor cells by metformin is negligible. It was also confirmed by immunohistochemistry (IHC) of tumors. Tumors of mice treated with metformin showed decreased expression of Ki67 as a proliferation marker, accordingly, increased expression of active caspase 3 as an