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O PAPEL MODULADOR DO RECEPTOR SÍMILE A TOLL 2 (TLR2) E DA MICROBIOTA INTESTINAL NA SENSIBILIDADE E SINALIZAÇÃO DA INSULINA EM CAMUNDONGOS

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Andrea Moro Caricilli

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Dedicatória

Dedico esta tese ao meu pai, que me ensinou a estudar o mundo, a conhecê-lo e fazer-lhe perguntas. De nada vale o esforço e o bom desempenho, se for esquecido o viver. De nada vale o ápice, se os arredores do percurso não tiverem sido conhecidos. De nada vale, se não se buscar a fortaleza por entre as fragilidades da vida.

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"(...) não é permitido consumar o que se almeja, porque não há aceitação de que o cor-de-rosa possa ser também truculento. E a constatação é a de que não se alcança o entendimento completo porque há a necessidade inerente de classificação, de segregação, de partidos concorrentes – como se houvesse impossibilidade de um só ser múltiplo e antitético. E é por isso que eu continuo, como os senhores, ora sendo jovem observador, ora loura Alice, ora aranha para tentar compreender os fios intrincados. Algumas vezes, inclusive, sou os próprios fios. Com isso, em minhas constantes mudanças de referencial, desfaço-me e refaço-me, transformando-me em um, em outro, em todos nós separadamente, até que a casa esteja enfim pronta e se aceite o truculento dentro do cor-de-rosa."

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Epígrafe

“Entender é sempre limitado. Mas não entender pode não ter fronteiras.” – Clarice Lispector

Resumo

Fatores ambientais e genéticos do hospedeiro interagem para controlar a microbiota intestinal, que pode ter um papel no desenvolvimento da obesidade e da resistência à insulina. Camundongos deficientes em TLR2, sob condições livres de microorganismos, estão protegidos da resistência à insulina induzida por dieta. Inibição aguda do TLR2 (4 dias de tratamento) com oligonucleotídeo antisense em camundongos alimentados com dieta hiperlipídica leva a um aumento da sensibilidade e da sinalização da insulina em tecido adiposo e muscular. É possível que a presença da microbiota intestinal possa reverter o fenótipo de um animal, induzindo resistência à insulina em um animal geneticamente determinado a ter aumento da sensibilidade à insulina, tal como o camundongo deficiente para TLR2. No presente estudo, nós investigamos a influência da microbiota intestinal nos parâmetros metabólicos, tolerância à glicose, sensibilidade e sinalização da insulina em camundongos deficientes para TLR2. A microbiota intestinal foi investigada (por metagenômica), as características metabólicas e a sinalização da insulina em camundongos deficientes para TLR2 em um biotério convencional. Os resultados mostraram que a perda do TLR2 em camundongos de biotério convencional resulta em fenótipo semelhante ao da síndrome metabólica, caracterizado por diferenças na microbiota intestinal, com um aumento de três vezes na proporção de Firmicutes e um pequeno aumento na de Bacteroidetes, em comparação com os controles. Essas alterações na microbiota foram acompanhadas por um aumento na absorção de LPS, inflamação subclínica, resistência à insulina, intolerância à glicose e posterior obesidade. Essa seqüência de eventos foi reproduzida em camundongos do tipo selvagem por transplante de

microbiota intestinal e revertida pelo tratamento com antibióticos. Em nível molecular, o mecanismo demonstrou-se único, com ativação do TLR4, associado com estresse de retículo endoplasmático e ativação da JNK, sem, porém, ativação da via IKKβ-IκB-NFκB. Nossos resultados também mostraram que, em camundongos deficientes para TLR2, houve redução de células T regulatórias em tecido adiposo visceral, sugerindo que essa regulação pode contribuir para a resistência à insulina nesses animais. Nesse sentido, nossos resultados enfatizam o papel da microbiota na complexa rede de interações moleculares e celulares que ligam genótipo e fenótipo, e suas potenciais implicações em alterações humanas envolvendo obesidade, diabetes e outras doenças imunológicas.

Abstract

Environmental factors and host genetics interact to control the gut microbiota, which may have a role in the development of obesity and insulin resistance. TLR2 deficient mice, under germ-free conditions are protected from diet-induced insulin resistance. Diet-induced obese mice, acutely treated with TLR2 oligonucleotide antisense during 4 days showed increased insulin sensitivity and signaling in muscle and white adipose tissue. It is possible that the presence of gut microbiota could reverse the phenotype of an animal, inducing insulin resistance in an animal genetically determined to have increased insulin sensitivity, such as the TLR2 KO mice. In the present study, we investigated the influence of gut microbiota on metabolic parameters, glucose tolerance, insulin sensitivity and signaling of TLR2-deficient mice. We investigated the gut microbiota (by metagenomics), the metabolic characteristics and insulin signaling in TLR2 knockout (KO) mice in a non-germ free-facility. Results showed that the loss of TLR2 in conventionalized mice results in a phenotype reminiscent of metabolic syndrome, characterized by differences in the gut microbiota, with a 3-fold increase in Firmicutes and a slight increase in Bacteroidetes compared with controls. These changes in gut microbiota were accompanied by an increase in LPS absorption, subclinical inflammation, insulin resistance, glucose intolerance and, later, obesity. In addition, this sequence of events was reproduced in WT mice by microbiota transplantation and was also reversed by antibiotics. At molecular level the mechanism was unique with activation of TLR4, associated with ER stress and JNK activation, but no activation of the IKKβ-IκB-NFκB pathway. Our data also showed that in TLR2 KO mice there was a reduction in regulatory T cell in visceral

fat suggesting that this modulation may also contribute to the insulin resistance of these animals. Our results emphasize the role of microbiota in the complex network of molecular and cellular interactions that link genotype to phenotype and have potential implications for common human disorders involving obesity, diabetes and even other immunological disorders.

Lista de abreviaturas

Palavra por extenso	Abreviatura
Nocaute (*knockout*)	KO
Receptor símile a Toll	TLR
Diabetes mellitus	DM
quinase c-Jun N-terminal	JNK
Susbtrato do receptor de insulina	IRS
quinase inibitória de κB	IKK
Zonnula occludens-1	ZO-1
Tipo selvagem (*wild-type*)	WT
Lipopolissacarídeo	LPS
Co-ativador-1 'alfa' do receptor ativado por proliferador do peroxissoma	PGC-1α
Proteína quinase ativada por AMP	AMPK
Fator de necrose tumoral-alpha	TNF-α
Proteína inibitória κB	IκB
Fator de nuclear κB	NFκB
Interleucina	IL
Receptor de insulina	IR
Quinase serina-treonina	AKT
Dieta hiperlipídica	DH
Oligonucleotídeo antisense	ASO
Proteína quinase C	PKC
Transportador de glicose	GLUT
Small-interfering RNA	si-RNA

Sumário

[Introdução]

Introdução geral

A obesidade é reconhecida hoje como um dos mais importantes problemas de saúde pública em todo o mundo (1). Diversos estudos epidemiológicos e clínicos têm confirmado a contribuição da obesidade para o desenvolvimento e/ou avanço de diversas doenças, como hipertensão arterial e diabetes mellitus (2), além do aumento da mortalidade por complicações cardiovasculares.

A epidemia recente de obesidade e diabetes mellitus do tipo 2 (DM2) nos últimos 20 anos estimulou pesquisadores a investigarem os mecanismos responsáveis pelo desenvolvimento dessas doenças. A visão geral é a de que a obesidade e o DM2 têm uma causa genética e que são fortemente influenciados pelo ambiente (3-7).

A sinalização insulínica é desencadeada pela ligação da insulina ao receptor na membrana plasmática da célula. O receptor de insulina é um complexo heterodimérico que consiste de duas subunidades α, extracelulares e com capacidade de se ligar à insulina, e duas subunidades β, intra-celulares e com atividade de tirosina-quinase. A ligação da insulina às subunidades α leva à transfosforilação das β, ativando sua capacidade tirosina-quinase (8, 9).

Até agora, mais de 10 substratos para o receptor de insulina foram identificados, sendo quatro deles estruturalmente relacionados, chamados de proteínas-substrato do receptor de insulina (IRS) (10-14). Cada proteína-substrato contém um domínio de homologia “pleckstrin”, um domínio de ligação de proteína tirosina e numerosos resíduos de tirosina que sofrem fosforilação pelo receptor de tirosina quinase. As proteínas IRS variam no que tange à distribuição em tecidos e localização subcelular (15-18). Uma vez fosforilados,

esses substratos agem como moléculas-ancoradoras para proteínas que contêm domínios de homologia com Src – como é o caso da fosfatidil-inositol-3-quinase (PI 3-quinase) –, as quais, dessa maneira, tornam-se ativadas ou se associam a outras moléculas de sinalização, ligando uma complexa cascata de eventos. A fosforilação das proteínas IRSs cria sítios de ligação para a PI 3-quinase, promovendo sua ativação. Atualmente, a PI 3-quinase é a única molécula intracelular inequivocamente considerada essencial para o transporte de glicose (19). As proteínas-alvo conhecidas dessa enzima são quinase serina-treonina (Akt) e as isoformas atípicas da proteína quinase C (PKC) (ς e λ). A Akt é uma serina-quinase também envolvida na mobilização do transportador de glicose GLUT4 para a superfície celular e transporte de glicose (20).

Estudos nos últimos dez anos mostraram que a inflamação subclínica tem um importante papel no mecanismo molecular da resistência à insulina na obesidade e no DM2 (21-25), particularmente através das vias da quinase c-Jun N-terminal (JNK) e da quinase inibitória de κB (IKK) (22). A ativação dessas vias estimula a fosforilação inibitória de resíduos de serina do IRS-1 (26), reduzindo tanto a fosforilação em tirosina dos IRS-1 em resposta à insulina quanto à capacidade do IRS-1 de associar-se ao receptor de insulina e, por esse meio, inibe a sinalização e a ação da insulina (26-28).

A sobrevivência de organismos multicelulares depende de sua capacidade de combater infecções e curar danos, bem como depende da capacidade de estocar energia para momentos de baixa disponibilidade de nutrientes ou de necessidade de altas quantidades de energia. Assim, pode-se dizer que os sistemas metabólico e imunológico estão entre os requerimentos

básicos do reino animal; muitos sistemas nutricionais e patógeno-sensíveis são altamente conservados em organismos como *C. elegans* e *Drosophila* até os mamíferos. Não é surpresa, por conseguinte, que as vias metabólicas e imunológicas sejam extremamente inter-relacionadas. Muitos hormônios, citocinas, proteínas sinalizadoras, fatores de transcrição e lipídios bioativos têm funções tanto metabólicas quanto imunológicas. Por utilizarem a mesma maquinaria, observa-se um controle mútuo desses dois sistemas. Por exemplo, uma resposta inflamatória normal dá-se sobre o suporte metabólico, demandando uma redistribuição energética, particularmente uma mobilização lipídica, que desempenha importante função no combate à infecção durante a fase de resposta aguda (29). A resposta inflamatória básica, portanto, favorece o estado catabólico e suprime as vias anabólicas, tais como as vias conservadas da sinalização da insulina.

Recentemente, demonstrou-se que a ativação das vias JNK e IKK pode ser integrada na resistência à insulina através de receptores de membrana, como os receptores símiles a Toll (do inglês, *Toll-like receptors*) (TLRs) (30).

A ativação do TLR4 foi implicada na inflamação e na resistência à insulina induzidas por ácidos graxos saturados (30-34). O lipopolissacarídeo (LPS), um potente ligante do TLR4, induz a inflamação pelas vias JNK/AP1 e IKK/Fator de Necrose Tumoral κB (NFκB) em diversos tipos celulares (35-37). Recentemente, Shi e colegas (32) demonstraram uma atenuação da ativação da via NFκB e da resistência à insulina induzidas por lipídios em tecido adiposo de camundongos nocaute para TLR4. Vários outros estudos confirmaram que a deficiência de TLR4 protege os animais da inflamação induzida pela obesidade e da resistência à insulina (30, 33, 38). Além disso, foi demonstrado também

que camundongos deficientes em TLR4 ficam protegidos do ganho de peso e reduz-se a infiltração de macrófagos em tecido adiposo (39). Em contraste, as citocinas inflamatórias IL-6 e TNF-α mostraram-se aumentadas nesse tecido, sugerindo que vias pró-inflamatórias independentes do TLR4 podem existir.

Como o TLR4, a expressão do TLR2 está aumentada no tecido adiposo de indivíduos obesos e diabéticos (36, 40). Ácidos graxos saturados sinalizam através do TLR2 para a ativação das vias IKKβ/NFκB e JNK/AP-1 em miotubos e células epiteliais, respectivamente (41, 42). Observou-se maior associação de TLR2 com sua proteína adaptadora, MyD88, quanto maior a concentração de palmitato. A inativação (por anticorpo monoclonal antagonista do TLR2) e a diminuição da expressão de TLR2 nos miotubos (por siRNA) bloqueiam a produção de IL-6 e restauram a fosforilação induzida por insulina da Akt na presença de palmitato. Assim, em culturas de células, há evidências convincentes de que o TLR2, induzido por ácidos graxos saturados, promove resistência à insulina (41). Uma abordagem mais detalhada sobre a descrição dos TLRs será realizada em seguida, na página 18.

Nos últimos cinco anos, a literatura tem sugerido outros mecanismos para o desenvolvimento da obesidade e do DM2, os quais se encontram entre os fatores genéticos e os ambientais – trata-se da microbiota intestinal (36, 43-47). Dessa maneira, faz-se evidente que fatores genéticos e ambientais interagem para controlar a microbiota intestinal, que pode ter um papel no desenvolvimento dessas doenças (48).

Praticamente todos os organismos multicelulares vivem em íntima associação com microorganismos que os circundam, e seres humanos não constituem exceção. O corpo humano é habitado por um vasto número de

bactérias, archaea, vírus e eucariotos unicelulares. O conjunto de microorganismos que vivem em coexistência harmoniosa com seus hospedeiros é referido como microbiota, microflora ou flora normal (49, 50). Estima-se que a microbiota humana contenha aproximadamente 10^{14} células bacterianas, um número dez vezes maior do que o número de células humanas presentes (51, 52), e que o cólon contenha 70% dos microorganismos de todo o corpo (49, 52).

A maior parte da microbiota intestinal é composta por bactérias anaeróbicas estritas, que dominam as anaeróbicas facultativas e as aeróbicas por duas ou três ordens de magnitude (51, 53, 54). Embora haja mais de 50 filos bacterianos descritos até o momento (55), o trato gastrintestinal humano é dominado por dois filos: Bacteroidetes e Firmicutes, enquanto Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria e Cyanobacteria estão presentes em menores proporções (56).

Estudos metagenômicos demonstraram que a proporção de Firmicutes é maior em animais e humanos obesos, em comparação com os controles magros, e que isso se correlaciona com aumento do número de genes que codificam enzimas que lisam polissacarídeos da dieta que não seriam digeríveis de outra maneira, com mais produtos de fermentação e com menos calorias remanescentes nas fezes de indivíduos obesos (57, 58). Outro mecanismo pelo qual a microbiota pode contribuir para alterações metabólicas é desencadeando inflamação sistêmica (59).

Muitos estudos mostraram que o genótipo do hospedeiro pode influenciar na composição de sua microbiota intestinal. Por exemplo, proporções de grupos bacterianos majoritários no intestino murino encontram-

se alteradas em camundongos geneticamente obesos, comparados com seus controles magros (60). Outras investigações mostraram que a dieta pode afetar a composição da microbiota intestinal. O consumo de uma dieta rica em lipídios, que induz o ganho de peso, é capaz de alterar de forma significativa a composição da microbiota no trato gastrintestinal murino (61). Manipulações dietéticas que limitam o ganho de peso foram também capazes de reverter os efeitos da obesidade induzida por dieta na microbiota.

O sistema imunológico coevolui com a microbiota durante a vida pós-natal, o que permite que o hospedeiro e a microbiota coexistam em um relacionamento de benefício mútuo (62, 63). A importância da microbiota intestinal no desenvolvimento tanto do sistema imune de mucosa intestinal quanto do sistêmico pode ser prontamente observada em estudos com camundongos livres de microorganismos. Estes animais apresentam um número anormal de diversos tipos celulares e produtos de células imunes, bem como deficiências em estruturas linfóides locais ou sistêmicas. Baços e linfonodos de camundongos livres de microorganismos são parcamente formados. Esses animais possuem também hipoplasia de placas de Payer (64) e uma redução do número de folículos linfóides maduros (65). O seu número de células produtoras de IgA é reduzido, bem como os níveis de imunoglobulinas secretadas (tanto IgA quanto IgG) (64). Eles também exibem irregularidades nos níveis e nos perfis de citocinas (66) e prejuízo na geração da tolerância oral (67).

Demonstrou-se que uma complexa comunidade microbiana contendo uma significativa proporção de Bacteroidetes é requerida para a diferenciação de células pró-inflamatórias Th17 (68). De maneira interessante, a colonização

de camundongos livres de microorganismos com flora de Schaedler alterada foi insuficiente para promover a diferenciação de células Th17, apesar do fato de a flora de Schaedler alterada conter um número de bactérias do filo Bacteroidetes (69). Esses achados ressaltam a complexidade de interações entre hospedeiro, microbiota e comunidades da microbiota, indicando que a cooperação entre membros da microbiota pode ser requerida para promover o desenvolvimento normal do hospedeiro.

Por outro lado, o trato gastrintestinal precisa coexistir com o denso revestimento de bactérias sem induzir uma ativação imune excessiva e prejudicial. A prevenção dessa resposta pode ser alcançada tanto pela separação física entre bactérias e células hospedeiras, por modificações de antígenos quanto pela modulação de respostas imunes na direção da tolerância imunológica (70). Células imunes residentes do trato gastrintestinal normalmente possuem um fenótipo distinto das células de iguais linhagens encontradas sistemicamente. Por exemplo, células dendríticas encontradas na mucosa intestinal induzem preferencialmente a diferenciação de células T em Th2 (71) e T regulatórias (72), promovendo o estado tolerogênico no trato gastrintestinal.

Normalmente, o epitélio intestinal age como uma barreira contínua para evitar a translocação do LPS, componente da parede de bactérias gram-negativas, embora alguns eventos endógenos ou exógenos possam alterar essa função protetora. Entre os elementos que promovem o aumento da permeabilidade intestinal ("*leaky gut*"), e, por conseguinte, o aumento dos níveis plasmáticos de LPS, estão o consumo de álcool (73-76), o estresse por imobilização (77, 78) e a radiação (79). Além disso, outros estudos têm

mostrado que a modulação da microbiota intestinal a partir da administração de dieta hiperlipídica aumenta fortemente a permeabilidade intestinal, reduzindo-se a expressão de genes que codificam duas proteínas de junção celular, a *zonnula-occludens* (ZO)-1 e a ocludina, o que é comprovado com o tratamento com antibióticos, que restauram a integridade epitelial intestinal (44).

O sistema imune inato, particularmente, mostrou-se como um importante regulador da microbiota intestinal. O reconhecimento de padrões moleculares associados a microorganismos pelo sistema imune inato é realizado por receptores específicos, notavelmente pela família dos TLRs (80, 81).

Os TLRs são receptores de membrana do tipo I evolutivamente conservados, e seus homólogos são encontrados em plantas e animais. O domínio extracelular dos TLRs é rico em repetições de leucina, enquanto o domínio citoplasmático mostra grande homologia com os receptores de interleucina, sendo, assim, referido como domínio Toll/IL-1R (TIR). O gene Toll foi inicialmente identificado como um gene essencial ao desenvolvimento dorso-ventral do embrião de Drosophila. Posteriormente, a sinalização do Toll foi relacionada com respostas imunes contra infecções fúngicas (82). Essa descoberta levou à identificação de homólogos do Toll em mamíferos, sugerindo que os TLRs participassem também da imunidade inata nesses animais. Assim, observou-se que os TLRs eram essenciais ao reconhecimento de microorganismos.

Estudos recentes mostram que os TLRs, ativados em tecidos afetados da maioria das desordens inflamatórias, podem mediar a conexão entre sistema imunológico e metabolismo corpóreo (80). Demonstrou-se que o TLR4, sensor para LPS de bactérias gram-negativas, está envolvido na expressão de

citocinas pró-inflamatórias em macrófagos, adipócitos e fígado (36, 83). Nosso grupo e outros mostraram que camundongos geneticamente deficientes para TLR4 ou com uma mutação inativadora para esse receptor apresentam proteção contra resistência à insulina induzida por obesidade (30, 32). De modo semelhante, camundongos geneticamente deficientes para TLR2 estão protegidos da resistência à insulina induzida por dieta hiperlipídica (84, 85). Estudos de nosso laboratório mostraram também que inibição aguda da expressão de TLR2, pelo uso de oligonucleotídeo antisense para TLR2 durante quatro dias, também protege camundongos da resistência à insulina, melhorando a sinalização da insulina em músculo e tecido adiposo, quando os animais são submetidos à dieta hiperlipídica (86).

Vale destacar que o TLR2 foi descrito como também envolvido na sinalização por LPS (87), além do TLR4. Evidências sugerem que o TLR2 humano interage com CD14 para formar um complexo com o receptor de LPS. Além disso, descreve-se que existe uma cooperação na sinalização de TLR4 e TLR2. Essa cooperação é evidenciada quando se injeta LPS em camundongos KO para TLR2. Após o primeiro bolus de LPS, os camundongos KO para TLR2 mostram um sinal robusto para genes que codificam proteínas do sistema imune inato no cérebro. Porém, uma segunda infusão de LPS falha no desencadeamento da expressão de TNF-α em camundongos KO para TLR2. Esses resultados indicam que o TLR2 está envolvido na segunda onda de expressão de TNF-α após estímulo com LPS, e que há uma elegante cooperação entre TLR2 e TLR4 (88).

Por outro lado, camundongos geneticamente deficientes em TLR5 apresentam hiperfagia e desenvolvem características marcantes de síndrome

metabólica, incluindo hiperlipidemia, hipertensão, resistência à insulina e aumento da adiposidade (89), sendo essas doenças conseqüência de alterações na microbiota intestinal. É importante ressaltar que os estudos com camundongos geneticamente deficientes para TLR4 e TLR5 foram realizados sem a existência de condições livres de microorganismos (30, 32, 89), sugerindo que a microbiota tem uma importante influência no fenótipo dos camundongos deficientes para TLR5, induzindo obesidade e resistência à insulina; entretanto, em camundongos deficientes para TLR4, a microbiota não parece ter a mesma importância nesses fenômenos, dado que os camundongos ficam protegidos da resistência à insulina induzida por dieta, independente de o biotério ser ou não livre de microorganismos (30, 32).

Juntos, esses achados sugerem que a interação do sistema immune inato com a microbiota intestinal pode determinar a sensibilidade à insulina de um animal, e que os TLRs podem ter papéis diferentes nesse processo. O primeiro estudo desta dissertação mostra que o TLR2 é relevante na conexão entre metabolismo e imunidade inata, dado que sua inibição aguda com oligonucleotídeo antisense levou a um aumento da sensibilidade e sinalização da insulina em músculo e tecido adiposo de camundongos alimentados com dieta hiperlipídica. Em outros estudos com camundongos deficientes para TLR2, observa-se também aumento da sensibilidade e sinalização da insulina. Porém, como na maioria deles a microbiota não foi investigada, não é possível predizer a influência da mesma na proteção ou no desenvolvimento da resistência à insulina nesses camundongos. É possível que a presença de uma microbiota intestinal diferente possa reverter completamente o fenótipo de um animal, induzindo a resistência à insulina em um animal geneticamente

determinado a ter maior sensibilidade à insulina, como os deficientes, também chamados de nocaute (KO), para TLR2. Assim, o objetivo do presente estudo foi investigar a influência da microbiota intestinal em parâmetros metabólicos, tolerância à glicose, sensibilidade e sinalização da insulina em camundongos deficientes para TLR2.

[Objetivos 1]

Objetivos

Inhibition of toll-like receptor 2 expression improves insulin sensitivity and signaling in muscle and white adipose tissue of mice fed a high-fat diet

Os objetivos do presente trabalho consistiram em:

- Investigar a expressão do receptor TLR2 em músculo e tecido adiposo epididimal de camundongos alimentados com dieta hiperlipídica;

- Estudar o efeito da inibição do receptor TLR2 em camundongos alimentados com dieta hiperlipídica através do uso de oligonucleotídeo antisense para TLR2.

DECLARAÇÃO

A cópia do artigo de minha autoria, já publicado na revista Journal of Endocrinology, que consta de minha Dissertação de Doutorado, intitulada "O papel modulador do receptor símile a Toll 2 (TLR2) e da microbiota intestinal na sensibilidade e sinalização da insulina em camundongos", não infringe os dispositivos da Lei nº 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 15 de Fevereiro e 2012

Andrea Moro Caricilli

RG: 43.748.125-6

Prof. Dr. Mario José Abdalla Saad

RG: 13.614.299-0

[Objetivos 2]

Objetivos

Gut Microbiota Is a Key Modulator of Insulin Resistance in TLR 2 Knockout Mice

Os objetivos do presente trabalho consistiram em:

- Caracterizar a microbiota intestinal do camundongo KO para TLR2 de nosso biotério;

- Investigar a influência da composição da microbiota intestinal dos camundongos KO para TLR2 nos parâmetros metabólicos, tolerância à glicose, sensibilidade e sinalização da insulina.

DECLARAÇÃO

A cópia do artigo de minha autoria, já publicado na revista PLoS Biology, que consta de minha Dissertação de Doutorado, intitulada "O papel modulador do receptor símile a Toll 2 (TLR2) e da microbiota intestinal na sensibilidade e sinalização da insulina em camundongos", não infringe os dispositivos da Lei nº 9.610/98, nem o direito autoral de qualquer editora.

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[Capítulo 1]

Inhibition of toll-like receptor 2 expression improves insulin sensitivity and signaling in muscle and white adipose tissue of mice fed a high-fat diet

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Abstract

The aims of the present study were to investigate the expression of toll-like receptor 2 (TLR2) in muscle and white adipose tissue (WAT) of diet-induced obesity (DIO) mice, and also the effects of its inhibition, with the use of TLR2 antisense oligonucleotide (ASON), on insulin sensitivity and signaling. The expression of TLR2 was increased in muscle and WAT of DIO mice, compared with those that received standard chow. Inhibition of TLR2 in DIO mice, by TLR2 ASON, improved insulin sensitivity and signaling in muscle and WAT. In addition, data show that the inhibition of TLR2 expression prevents the activation of IKBKB, MAPK8, and serine phosphorylation of IRS1 in DIO mice, suggesting that TLR2 is a key modulator of the crosstalk between inflammatory and metabolic pathways. We, therefore, suggest that a selective interference with TLR2 presents an attractive opportunity for the treatment of insulin resistance in obesity and type 2 diabetes.

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Introduction

Obesity is associated with insulin resistance and a state of abnormal inflammatory response (Kadowaki *et al.* 2003, Bray 2004, Tsukumo *et al.* 2007). There is convincing evidence that the activation of the MAPK8, IKK, and NOS2 (Perreault & Marette 2001, Yuan *et al.* 2001, Hirosumi *et al.* 2002, Lee *et al.* 2003, Carvalho-Filho *et al.* 2005) pathways is related to the reduction in insulin sensitivity, but it has only recently been shown that these pathways may be integrated to cause insulin resistance by the activation of membrane receptors, such as toll-like receptors (TLRs; Aderem & Ulevitch 2000, Shi *et al.* 2006, Tsukumo *et al.* 2007).

There are at least 11 members of the TLR family in humans and 13 in mice (Takeda & Akira 2004). TLRs play a crucial role in the recognition of invading pathogens and the activation of subsequent immune responses against them. Individual TLRs recognize distinct pathogen-associated molecular patterns. The TLR family harbors an extracellular leucine-rich repeat domain, as well as a cytoplasmic domain that is homologous to that of the interleukin-1 receptor (IL1R1). Upon stimulation, TLR recruits IL1R1-associated protein kinases via adaptor MYD88, and finally induces the activation of nuclear factor-κB and mitogen-activated protein kinases, as well as the expression of inflammatory cytokines (Aderem & Ulevitch 2000, Heldwein & Fenton 2002, Akira & Sato 2003). Preliminary studies have indicated that loss-of-function mutation and knockout in TLR4 prevents insulin resistance induced by obesity or free fatty acids, suggesting an important role of TLR4 in the interface of innate immune system and energetic metabolism (Shi *et al.* 2006, Song *et al.* 2006, Kim *et al.* 2007, Nguyen *et al.* 2007, Poggi *et al.* 2007, Poulain-Godefroy & Froguel 2007, Tsukumo *et al.* 2007).

A recent study showed that palmitate treatment of differentiated C2C12 myotubes resulted in a time-dependent inhibition of insulin-activated signal transduction, through TLR2 activation (Senn 2006). However, the role of TLR2 in insulin resistance induced by a high-fat diet in animals has not yet been investigated.

The aims of the present study were to investigate the expression of TLR2 in muscle and white adipose tissue (WAT) of diet-induced obesity (DIO) mice, and also the effects of its inhibition, by the use of TLR2 antisense oligonucleotide (ASON), on insulin sensitivity and signaling.

Research design and Methods

Materials

Male Swiss mice were provided by the State University of Campinas Central Breeding Center (Campinas, São Paulo,

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Brazil). Human recombinant insulin was obtained from Eli Lilly. Routine reagents were purchased from Sigma Chemical, unless specified elsewhere.

Animals

All experiments were approved by the Ethics Committee at the State University of Campinas. Six-week-old Swiss mice were divided into two groups with similar body weights (20±2 g) and assigned to receive standard rodent chow and water *ad libitum* or a high-fat diet (DIO), consisting of 55% calories from fat, 29% from carbohydrate, and 16% from protein. Mice had free access to the diets for 2 months. Body weight was measured weekly. The insulin tolerance test was performed on these mice after 8 weeks on the diets, as described previously (Perreault & Marette 2001, Carvalho-Filho *et al.* 2005). Serum insulin was determined by RIA (Scott *et al.* 1981).

TLR2 expression in gastrocnemius muscle of DIO mice

After a period of 2 months, TLR2 expression in muscle and WAT was analyzed. Abdominal cavities of anesthetized mice were opened and fragments of gastrocnemius muscle and WAT were excised and immediately homogenized in solubilization buffer at 4 °C. Later, the samples were submitted to the direct immunoblotting process, using TLR2 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

TLR2 antisense (ASON) treatment of mice

After the period of 2 months, the mice that received DIO were divided into two groups, similar to those who received standard rodent chow: one group received an i.p. injection of 4·0 nmol TLR2 ASON (5′-GAG CTC GTA GCA TCC TCT-3′), and another received, by the same means, 4·0 nmol TLR2 nonsense (NSON; 5′-GCT CTA TGA CTC CCA G-3′), for 4 days up to 2 h before killing.

Insulin tolerance test (ITT)

Immediately before the first day of TLR2 ASON treatment, mice were submitted to an ITT, and again at 4 days after TLR2 ASON treatment. Insulin (1·5 U/kg) was administered by i.p. injection and blood samples were collected at 0, 5, 10, 15, 20, 25, and 30 min to determine serum glucose. The constant rate for glucose disappearance (K_{itt}) was calculated using the formula $0{\cdot}693/t_{1/2}$. Glucose $t_{1/2}$ was calculated from the slope of the least-squares analysis of plasma glucose concentrations during the linear decay phase (Bonora *et al.* 1989).

Insulin signaling

After the fourth day of TLR2 ASON treatment, mice were anaesthetized by i.p. injection of sodium thiopental and were used 10–15 min later, i.e., as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Five minutes after the insulin injection (1·5 U/kg i.p.), muscle and WAT were removed, minced coarsely, and homogenized immediately in extraction buffer, as described elsewhere (Thirone *et al.* 2004). Protein extracts obtained from the tissue were used for immunoblotting to analyze the insulin signaling.

Immunoprecipitation and immunoblotting

Abdominal cavities of anesthetized mice were opened and fragments of gastrocnemius muscle and WAT were excised and immediately homogenized in solubilization buffer at 4 °C (1% Triton X-100, 100 mmol/l Tris–HCl (pH 7·4), 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium orthovanadate, 2·0 mmol/l phenylmethylsulphonyl fluoride, and 0·1 mg aprotinin/ml) with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA), operated at a maximum speed of 30 s. Insoluble material was removed by centrifugation for 40 min at 11 000 r.p.m. in a 70.Ti rotor (Beckman, Fullerton, CA, USA) at 4 °C. The protein concentration of the supernatants was determined by the Bradford dye binding method (Bradford 1976). Aliquots of the resulting supernatants containing 1·0 mg total protein were used for immunoprecipitation with antibodies against TLR2 at 4 °C overnight, followed by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with MYD88 antibodies.

In direct immunoblotting experiments, 150 μg protein extracts, obtained from gastrocnemius muscle were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-TLR, anti-phospho [Ser-307] IRS1, anti-phospho [Tyr-941] IRS1, anti-NFKBIB, anti-phospho-AKT, anti-phospho-IKKα/β, anti-phospho-MAPK8, and anti-β-actin (Santa Cruz Biotechnology).

In both immunoprecipitation and immunoblotting experiments, proteins were denaturated by boiling in Laemmli buffer (Laemmli 1970). For immunoblotting, the sample buffer contained 100 mM dithiothreitol (DTT), whereas for immunoprecipitation, 50 mM DTT was used. Specific bands were labeled with a chemioluminescence kit (Sigma) and visualization was performed by exposure of the membranes to X-ray films. Band intensities were quantified by digital densitometry (UN-SCAN-IT gel 6·1; Silk Scientific Inc., Orem, UT, USA) of the developed autoradiographs.

Homeostasis model assessment

The degree of insulin resistance and β-cell function were estimated by the homeostasis model assessment of insulin resistance (HOMA-IR), as described by Matthews *et al.* (1985). HOMA-IR was calculated by the formula: fasting plasma glucose (mmol/l)×fasting plasma insulin (mU/l)/22·5.

Statistical analysis

Data are expressed as means±S.E.M., and the number of independent experiments is indicated. For statistical analysis,

the groups were compared using a two-way ANOVA with the Bonferroni test for *post hoc* comparisons. The level of significance adopted was $P<0{\cdot}05$.

Results

Animal characteristics and effect of TLR2 ASON

The infusion of TLR2 ASON for 4 days did not change the plasma glucose or the serum insulin levels in control animals (fed standard rodent chow; Fig. 1A and B). As expected, plasma glucose and serum insulin levels were higher in DIO mice, and when these animals were treated with TLR2 ASON, there was a decrease in serum insulin levels (Fig. 1B). The glucose disappearance rate during the ITT was lower in DIO mice, reinforcing the suggestion that these animals presented insulin resistance. The administration of TLR2 NSON to controls mice had no effect on insulin sensitivity, as measured by the ITT. In DIO mice treated with TLR2 ASON, there was an increase in glucose disappearance rate, compared with those without TLR2 ASON treatment, suggesting that inactivating TLR2 improves insulin sensitivity in these mice (Fig. 1C). This improvement is also suggested by the HOMA-IR, which is increased in DIO mice and significantly decreased in those treated with TLR2 ASON (Fig. 1D).

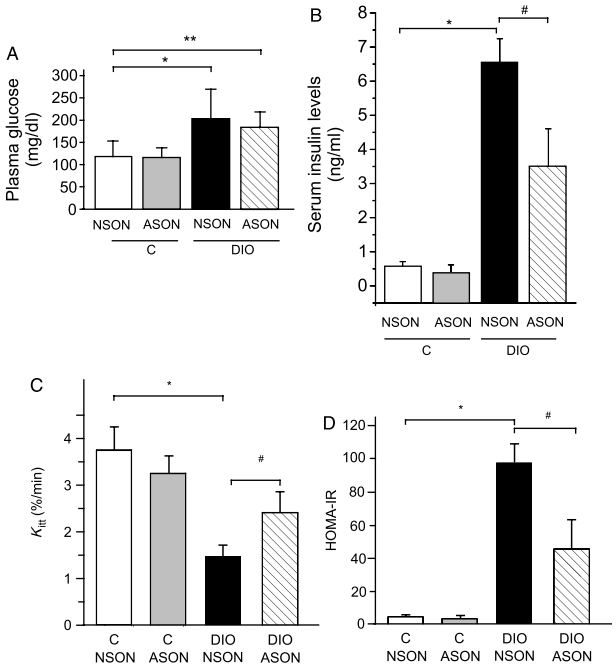


Figure 1 Plasma glucose and serum insulin levels. Bars represent mean±S.E.M. from six to eight mice. (A) Plasma glucose levels are higher in DIO mice compared with those fed on standard chow. However, there is no significant difference between TLR2 NSON and TLR2 ASON treatments. (B) Serum insulin levels are increased in DIO mice treated with TLR2 ASON mice. (C) Insulin sensitivity obtained from an ITT. (D) HOMA-IR, calculated by the formula: fasting plasma glucose (mmol/l) × fasting plasma insulin (mU/l)/22·5. *$P<0{\cdot}05$ between NSON TLR2-treated control mice and NSON TLR2-treated DIO mice. **$P<0{\cdot}05$ between NSON TLR2-treated control mice and TLR2 ASON-treated DIO mice. #$P<0{\cdot}05$ between TLR2 ASON- and TLR2 NSON-treated DIO mice. ITT, insulin tolerance test; HOMA-IR, homeostasis model assessment-insulin resistance.

Effect of TLR2 ASON on TLR2 expression and downstream signaling in gastrocnemius muscle

Administration of TLR2 ASON for 4 days reduced TLR2 expression by 50–60% in the muscle of mice fed on standard chow. An increased expression of TLR2 was seen in the gastrocnemius muscle of mice that received DIO, compared with those that received standard rodent chow (Fig. 2A). Obese animals treated with TLR2 ASON showed a marked decrease in TLR2 expression in the muscle, indicating that the oligonucleotide was efficient for its purpose (Fig. 2A).

In order to investigate TLR2 activation, the extracts from gastrocnemius muscle were submitted to immunoprecipitation using anti-TLR2 antibody, and then to immunoblotting using anti-MYD88 antibody, since the MYD88 protein binds to TLR2 when its ligand is recognized (Tohno *et al.* 2007). There was a significant decrease in TLR2/MYD88 interaction in control mice treated with TLR2 ASON. The co-immunoprecipitation of TLR2/MYD88 was observed to increase in the muscle of DIO mice compared with control mice, but when those obese mice were treated with TLR2 ASON, the co-immunoprecipitation of TLR2/MYD88 decreased in the muscle (Fig. 2B). These data suggest that TLR2 activation is increased in the muscle of DIO mice and decreased in those treated with TLR2 ASON.

Downstream from TLR2 activation, activation of the IKK/NFKBIB/NF-κB pathway occurs. The activation of IKK was investigated by the detection of its phosphorylation on serine 181. Mice fed on standard chow did not demonstrate a significant difference in IKK phosphorylation when treated with TLR2 NSON or ASON. As expected, the muscle of DIO mice presented an increased phosphorylation of IKK, while the DIO mice treated with TLR2 ASON demonstrated a decreased phosphorylation of this protein. Total IKBKB protein expression was the same in all samples (Fig. 2C).

IKK activation was also monitored using NFKBIB protein abundance. In mice fed on standard chow, no statistically significant differences were seen between the treatment with TLR2 NSON or TLR2 ASON. Our results showed a reduction in NFKBIB protein expression in the muscle of DIO mice, suggesting that there is an activation of IKK. However, after TLR2 ASON treatment, there is a complete recovery of NFKBIB expression in this tissue. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody (Fig. 2D).

MAPK8 activation was determined by monitoring its phosphorylation (Thr183 and Tyr185). In mice fed a standard chow, the activation of MAPK8 was the same in both treatments, with TLR2 NSON and with TLR2 ASON. In a

manner similar to IKK, MAPK8 phosphorylation was increased in the muscle of mice on a DIO, but decreased in those treated with TLR2 ASON. Total MAPK8 protein expression was the same in all samples (Fig. 2E).

Effect of TLR2 ASON on insulin signaling in gastrocnemius muscle

In mice fed on standard chow, treatment with TLR2 ASON did not change insulin-induced IRS1 tyrosine phosphorylation compared with TLR2 NSON-treated mice. Insulin-induced IRS1 tyrosine phosphorylation was reduced in the muscle of DIO mice, and treatment with TLR2 ASON partially reversed this alteration. Total IRS1 protein expression was the same in all samples (Fig. 3A). Insulin-induced AKT phosphorylation was also reduced in the muscle of DIO mice. However, when these mice were treated with TLR2 ASON, there was an improvement in insulin-induced AKT phosphorylation. Total AKT protein expression was the same in all samples. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody (Fig. 3B).

It is known that the activation of IKK and MAPK8 can induce insulin resistance, at least in part, through phosphorylation of IRS1 at Ser^{307} (Hirosumi *et al.* 2002). Mice fed a standard chow did not show any difference in the phosphorylation of IRS1 at Ser^{307} when treated with TLR2 ASON. In DIO mice, there was an increase in the phosphorylation of IRS1 at Ser^{307} in gastrocnemius muscle, and the treatment with TLR2 ASON partially reversed this alteration (Fig. 3C).

Effect of TLR2 ASON on TLR2 expression and downstream signaling in WAT

As seen in gastrocnemius muscle, administration of TLR2 ASON for 4 days reduced TLR2 expression by 50–60% in the WAT of mice fed on standard chow. An increased expression of TLR2 was seen in the WAT of mice that received DIO, compared with those that received standard rodent chow. Obese animals treated with TLR2 ASON showed, as expected, a decreased expression of TLR2 in WAT (Fig. 4A).

We also investigated the co-immunoprecipitation of TLR2/MYD88 in WAT and there was a significant decrease in TLR2/MYD88 interaction in control mice treated with TLR2 ASON. In DIO mice, when the samples were immunoprecipitated with TLR2 and blotted with MYD88, there was an increase in the band intensity compared with control mice. But when these mice were treated with TLR2 ASON, the co-immunoprecipitation of TLR2/MYD88 was decreased in WAT (Fig. 4B).

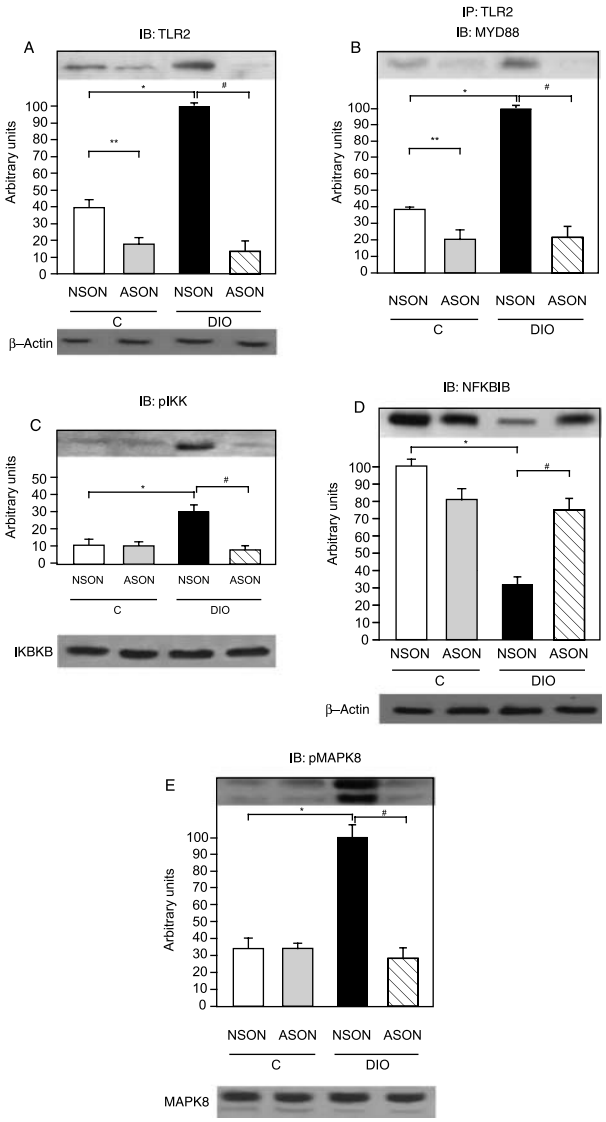
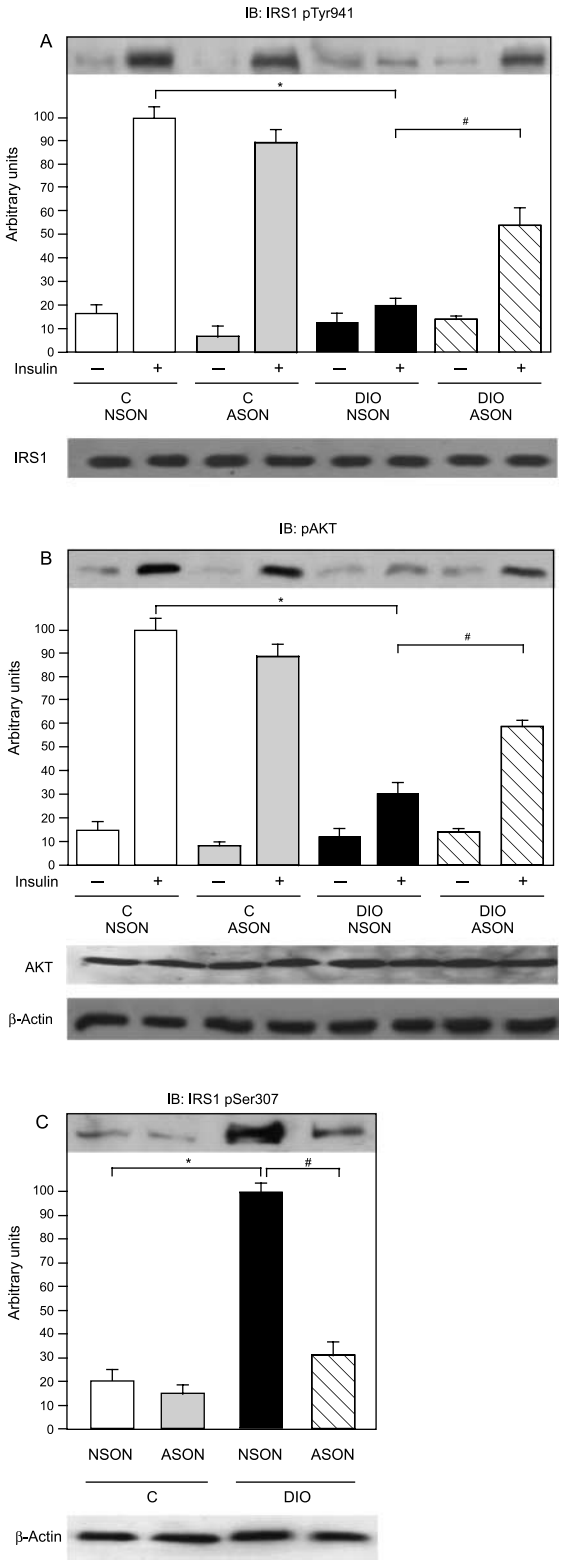


Figure 2 Effect of TLR2 ASON on TLR2 expression and downstream signaling in gastrocnemius muscle. Total muscle lysates were separated by western blotting and immunoblotted with the respective antibodies described in the Methods section ($n=6$ mice per group). Each bar represents the mean±S.E.M. of three experiments. Representative blots are shown. (A) Effects of TLR2 ASON treatment on TLR2 expression in the muscle of DIO mice. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody. (B) Co-immunoprecipitation of TLR2/MYD88. (C) IKK activation detected by its phosphorylation on serine 181. To determine the protein levels of IKBKB, the membranes were stripped and reprobed with anti-IKBKB. (D) IKK activation detected by an indirect method, the expression of NFKBIB. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody. (E) MAPK8 activation determined by monitoring its phosphorylation (Thr183 and Tyr185). To determine the protein levels of MAPK8, the membranes were stripped and reprobed with anti-MAPK8. *$P<0{\cdot}05$ between NSON TLR2-treated control mice and NSON TLR2-treated DIO mice. **$P<0{\cdot}05$ between NSON TLR2 and ASON TLR2 control mice. #$P<0{\cdot}05$ between TLR2 ASON- and TLR2 NSON-treated DIO mice.



Mice fed a standard chow did not show a significant difference when treated with TLR2 NSON or ASON with regard to phosphorylation of IKK. As expected, WAT of DIO mice presented an increased phosphorylation of IKK, while DIO mice treated with TLR2 ASON had a decreased phosphorylation of this protein. Total IKBKB protein expression was the same in all samples (Fig. 4C). Related to NFKBIB protein expression, in mice fed on standard chow, no statistically significant differences were seen between the treatment with TLR2 NSON or TLR2 ASON. Our results showed a reduction in NFKBIB protein expression in the WAT of DIO mice, suggesting that there is an activation of IKK. However, after TLR2 ASON treatment, there was a complete recovery of NFKBIB expression in this tissue. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody (Fig. 4D).

As seen in muscle, in mice fed on standard chow, the phosphorylation of MAPK8 did not change with TLR2 ASON treatment. On the other hand, MAPK8 phosphorylation was increased in the WAT of DIO mice, but decreased in those treated with TLR2 ASON. Total MAPK8 protein expression was the same in all samples (Fig. 4E).

Effect of the inhibition of TLR2 on insulin signaling in WAT

In mice fed on standard chow, treatment with TLR2 ASON did not change insulin-induced IRS1 tyrosine phosphorylation. Insulin-induced IRS1 tyrosine phosphorylation was reduced in the WAT of DIO mice, and treatment with TLR2 ASON partially reversed this alteration. Total IRS1 protein expression was the same in all samples (Fig. 5A). Insulin-induced AKT phosphorylation was also reduced in the WAT of DIO mice. However, when these mice were treated with TLR2 ASON, there was an improvement in insulin-induced AKT phosphorylation. Total AKT protein expression was the same in all samples. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody (Fig. 5B).

The phosphorylation of IRS1 at Ser^{307} in the WAT of mice fed a standard chow and treated with TLR2 ASON did not differ from that in TLR2 NSON-treated mice. As expected in

Figure 3 Effect of the inhibition of TLR2 on insulin sensitivity in gastrocnemius muscle. Total muscle lysates were separated by western blotting and immunoblotted with the respective antibodies described in the Methods section ($n=6$ mice per group). Each bar represents the mean ± S.E.M. of three experiments. Representative blots are shown. (A) Insulin-induced phosphorylation of IRS1 (Tyr941). To determine the protein levels of IRS1, the membranes were stripped and reprobed with anti-IRS1. (B) Insulin-induced serine phosphorylation of AKT. To determine the protein levels of AKT, the membranes were stripped and reprobed with anti-AKT. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody. (C) Ser^{307} phosphorylation of IRS1. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody. *$P<0{\cdot}05$ between NSON TLR2-treated control mice and NSON TLR2-treated DIO mice. #$P<0{\cdot}05$ between TLR2 ASON- and TLR2 NSON-treated DIO mice.

the WAT of DIO mice, there was an increase in the phosphorylation of IRS1 at Ser^{307}, and the treatment with TLR2 ASON partially reversed this alteration (Fig. 5C).

Discussion

The consumption of a high-fat diet is one of the major causes of obesity and insulin resistance. However, the molecular mechanism by which DIO leads to insulin resistance and a state of abnormal inflammatory response are not completely understood (Kadowaki *et al.* 2003, Barreiro *et al.* 2004, Bray 2004, Araujo *et al.* 2005, Prada *et al.* 2005, Tsukumo *et al.* 2007). Our data showed an increased expression of TLR2 in the muscle and WAT of DIO mice, and treatment with TLR2 ASON improves the insulin resistance of these mice, suggesting that TLR2 is an important modulator in the crosstalk between inflammation and metabolic pathway in DIO. However, in mice fed on standard chow, the inhibition of TLR2 did not cause an improvement in insulin sensitivity. This is probably due to the fact that TLR2 has a role in insulin resistance in obesity, but does not have a central role in the control of insulin sensitivity and signaling in physiological conditions.

A recent study showed that palmitate treatment of differentiated C2C12 myotubes results in a time-dependent inhibition of insulin-activated signal transduction, specifically tyrosine phosphorylation of the insulin receptor and the phosphorylation of AKT. Palmitate also induced the production of significant amounts of IL6 and the phosphorylation of p38 and MAPK8. Additionally, palmitate induced a rapid activation of TLR2 signal transduction in a time-dependent manner. Treatment of cells with a monoclonal antagonist antibody anti-TLR2 blocked the subsequent induction of IL6 production and restored the insulin-induced AKT phosphorylation (Senn 2006). Therefore, cell culture data provide convincing evidence that fatty acids leads to insulin resistance at least in part through TLR2.

Our data show that, in the muscle and WAT of DIO mice, there is an increase in TLR2 expression, a modulation that might have a role in insulin resistance in these animals. Upon stimulation, TLR recruits IL1R1-associated protein kinases via adaptor protein MYD88 and finally induces the activation of nuclear factor-κB and mitogen-activated protein kinases, as well as the expression of inflammatory cytokines (Aderem & Ulevitch 2000, Heldwein & Fenton 2002, Akira & Sato 2003). Our data demonstrate that, in the muscle and WAT from DIO mice, MYD88 associates with the TLR2, activating downstream kinases such as IKK, weakening insulin signal transduction, and reducing the glucose disappearance rate. The reduced insulin sensitivity of DIO mice, as demonstrated by reduced glucose disappearance rate, high insulin levels, and higher HOMA-IR, was improved when these mice were treated with TLR2 ASON.

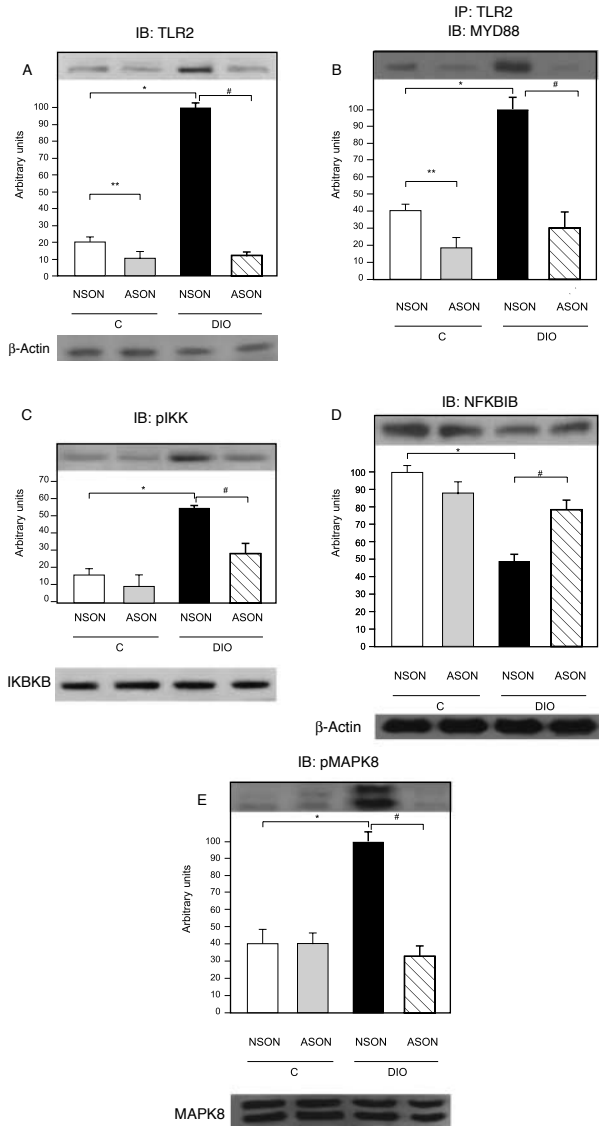
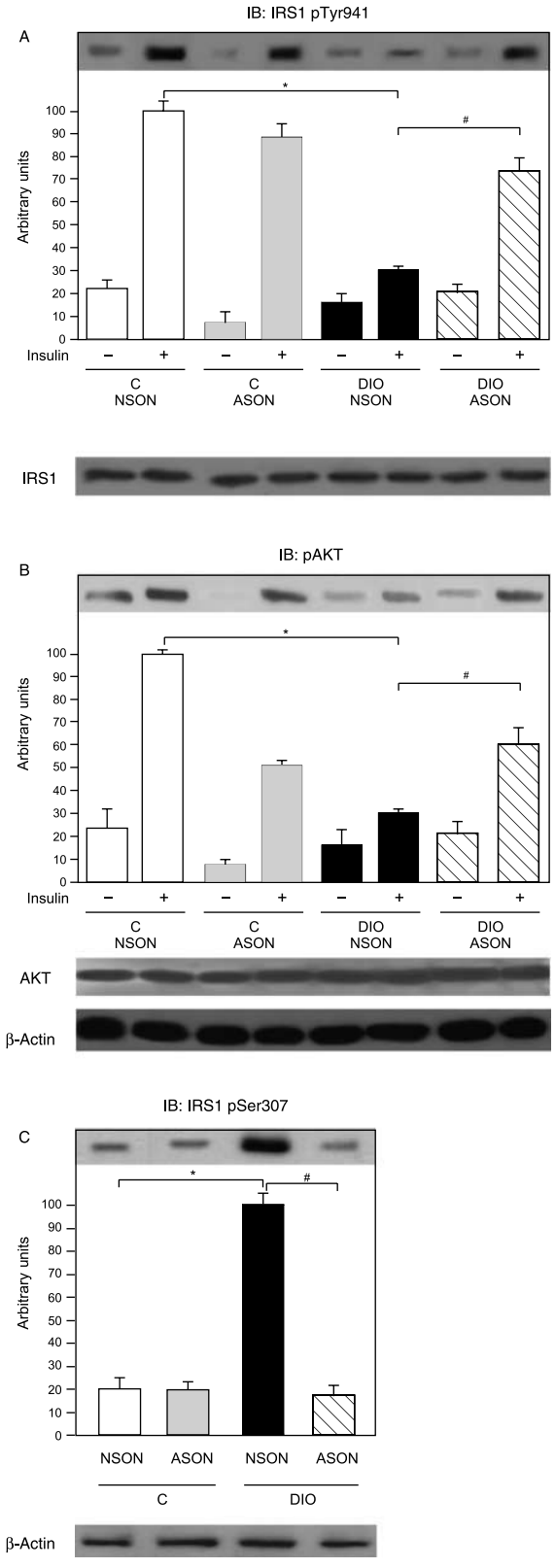


Figure 4 Effect of TLR2 ASON on TLR2 expression and downstream signaling in WAT. Total white adipose tissue lysates were separated by western blotting and immunoblotted with the respective antibodies described in the Methods section ($n=6$ mice per group). Each bar represents the mean ± S.E.M. of three experiments. Representative blots are shown. (A) Effects of TLR2 ASON treatment on TLR2 expression in the WAT of DIO mice. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody. (B) Co-immunoprecipitation of TLR2/MYD88. (C) IKK activation detected by its phosphorylation on serine 181. To determine the protein levels of IKBKB, the membranes were stripped and reprobed with anti-IKBKB. (D) IKK activation detected by an indirect method, the expression of NFKBIB. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody. (E) MAPK8 activation determined by monitoring its phosphorylation (Thr183 and Tyr185). To determine the protein levels of MAPK8, the membranes were stripped and reprobed with anti-MAPK8. *$P<0{\cdot}05$ between NSON TLR2-treated control mice and NSON TLR2-treated DIO mice. **$P<0{\cdot}05$ between NSON TLR2 and ASON TLR2 control mice. #$P<0{\cdot}05$ between TLR2 ASON- and TLR2 NSON-treated DIO mice.



The blunted insulin-stimulated IRS1 tyrosine (941) phosphorylation and phosphorylation of AKT in the muscle of DIO mice was restored in the DIO mice treated with TLR2 ASON, providing a biochemical correlate for increased *in vivo* insulin sensitivity. Serine phosphorylation of IRS1 has been proposed as a general mechanism of functional inhibition of the IRS1 protein, and Ser^{307} phosphorylation has become a molecular indicator of insulin resistance (Aguirre *et al.* 2002, Gao *et al.* 2002, Hirosumi *et al.* 2002, Lee *et al.* 2003). Ser^{307} phosphorylation was induced by the high-fat diet in the muscles and WAT of DIO mice, accompanied by a reduction in insulin-induced IRS1 tyrosine (941) phosphorylation levels. This regulation of IRS1, induced by the DIO, was rescued in the DIO mice treated with TLR2 ASON.

Ser^{307} is reported to be a phosphoacceptor of MAPK8 and IKK (Aguirre *et al.* 2002, Gao *et al.* 2002) and, as previously described (Yuan *et al.* 2001, Hirosumi *et al.* 2002), our results also show that both kinases are activated the in tissues of DIO controls. It is well known that the activation of TLR2 induces a complex signaling pathway that activates IKK and MAPK8 (Takeda *et al.* 2003). Our data, demonstrating that the inhibition of TLR2 expression rescues the tissues from the activation of IKK and MAPK8 and from the insulin resistance, suggest that TLR2 is a key modulator in the crosstalk between inflammatory and metabolic pathways. However, the fact that the activation of IKK leads to the insulin resistance is not uniformly observed (Polkinghorne *et al.* 2008).

In the past 2 years, different studies showed that TLR4 has an important role in obesity-induced insulin resistance, and that mice with a loss-of-function mutation or knockout for TLR4 are protected from diet-induced insulin resistance (Senn 2006, Shi *et al.* 2006, Song *et al.* 2006, Kim *et al.* 2007, Nguyen *et al.* 2007, Poggi *et al.* 2007, Poulain-Godefroy & Froguel 2007, Tsukumo *et al.* 2007). Our data show that TLR2 may also have a role in the interface of subclinical inflammation and insulin resistance in DIO mice.

In summary, our data, showing that the inhibition of TLR2 expression rescues the cells from the activation of IKK and MAPK8 and insulin resistance in the DIO mice, indicate that TLR2 is a key modulator in the crosstalk between

Figure 5 Effect of the inhibition of TLR2 on insulin sensitivity in WAT. Total white adipose tissue lysates were separated by western blotting and immunoblotted with the respective antibodies described in the Methods section ($n=6$ mice per group). Each bar represents the mean ± S.E.M. of three experiments. Representative blots are shown. (A) Insulin-induced phosphorylation of IRS1 (Tyr941). To determine the protein levels of IRS1, the membranes were stripped and reprobed with anti-IRS1. (B) Insulin-induced serine phosphorylation of AKT. To determine the protein levels of AKT, the membranes were stripped and reprobed with anti-AKT. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody. (C) Ser^{307} phosphorylation of IRS1. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody. $*P<0{\cdot}05$ between control mice and DIO mice. $\#P<0{\cdot}05$ between TLR2 ASON- and TLR2 NSON-treated DIO mice.

inflammatory and metabolic pathways. We, therefore, suggest that a selective interference with TLR2 presents an attractive opportunity for the treatment of obesity, insulin resistance, and type 2 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

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[Capítulo 2]

Gut Microbiota Is a Key Modulator of Insulin Resistance in TLR 2 Knockout Mice

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Abstract

Environmental factors and host genetics interact to control the gut microbiota, which may have a role in the development of obesity and insulin resistance. TLR2-deficient mice, under germ-free conditions, are protected from diet-induced insulin resistance. It is possible that the presence of gut microbiota could reverse the phenotype of an animal, inducing insulin resistance in an animal genetically determined to have increased insulin sensitivity, such as the TLR2 KO mice. In the present study, we investigated the influence of gut microbiota on metabolic parameters, glucose tolerance, insulin sensitivity, and signaling of TLR2-deficient mice. We investigated the gut microbiota (by metagenomics), the metabolic characteristics, and insulin signaling in TLR2 knockout (KO) mice in a non-germ free facility. Results showed that the loss of TLR2 in conventionalized mice results in a phenotype reminiscent of metabolic syndrome, characterized by differences in the gut microbiota, with a 3-fold increase in Firmicutes and a slight increase in Bacteroidetes compared with controls. These changes in gut microbiota were accompanied by an increase in LPS absorption, subclinical inflammation, insulin resistance, glucose intolerance, and later, obesity. In addition, this sequence of events was reproduced in WT mice by microbiota transplantation and was also reversed by antibiotics. At the molecular level the mechanism was unique, with activation of TLR4 associated with ER stress and JNK activation, but no activation of the IKKβ-IκB-NFκB pathway. Our data also showed that in TLR2 KO mice there was a reduction in regulatory T cell in visceral fat, suggesting that this modulation may also contribute to the insulin resistance of these animals. Our results emphasize the role of microbiota in the complex network of molecular and cellular interactions that link genotype to phenotype and have potential implications for common human disorders involving obesity, diabetes, and even other immunological disorders.

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Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: ASO, antisense oligonucleotide; CFU, colony forming units; HFD, high-fat diet; IR, insulin receptor; IRS, insulin receptor substrate; KO, knockout; PBA, phenyl butyric acid; PBS, phosphate buffered saline; RER, respiratory exchange ratio; rRNA, ribosomal RNA; T2DM, type 2 diabetes mellitus; TJ, tight junction; TLRs, Toll-like receptors; TLR2, Toll-like Receptor 2; ZO, zonula occludens

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Introduction

The recent epidemics of obesity and type 2 diabetes mellitus (T2DM) in the past 20 years have stimulated researchers to investigate the mechanisms that are responsible for the development of these diseases. The general view is that obesity and T2DM have a genetic background and are strongly influenced by the environment and that insulin resistance is an early alteration in these diseases [1–5]. In addition, studies over the past 10 years have also shown that subclinical inflammation has an important role in the molecular mechanism of insulin resistance in obesity and T2DM [6–10]. During the past five years, an increasing body of literature has suggested other components of the mechanisms of these diseases that lie between the genetic and the environment factors, where the gut microbiota are now considered to make an important contribution to these mechanisms [11–16]. Then, it is now clear that environmental factors and host genetics interact to control the gut microbiota, which may have a role in the development of obesity and insulin resistance [17].

Metagenomic studies demonstrated that the proportion of Firmicutes is higher in obese animals and in humans, compared with lean controls, and this correlates with a higher number of genes encoding enzymes that break down otherwise indigestible dietary polysaccharides, with more fermentation end products and fewer calories remaining in the faeces of obese individuals [18,19]. Another mechanism by which the microbiome may contribute to metabolic disorders is by triggering systemic inflammation [20]. The immune system coevolves with the microbiota during postnatal life, which allows the host and microbiota to coexist in a mutually beneficial relationship [21,22]. In particular, the innate immune system has emerged as a key regulator of the gut microbiota. Innate immune recognition of microbe-associated molecular patterns is

Author Summary

An intricate interaction between genetic and environmental factors influences the development of obesity and diabetes. Previous studies have shown that mice lacking an important receptor of the innate immune system, Toll-like Receptor 2 (TLR2), are protected from insulin resistance. Given that the innate immune system has emerged as a key regulator of the gut microbiota, we undertook to investigate in this study whether the gut microbiota have a role in modulating the response to insulin. By rearing these TLR2 mutant mice in conventional facilities (as opposed to "germ-free" conditions) we figured that they would develop an altered gut microbiota. In contrast to previous studies, our results show that these TLR2 mutant mice now develop a diseased phenotype reminiscent of metabolic syndrome, including weight gain, and end up with gut microbiota similar to that found in obese mice and humans. These mice could be rescued by treatment with broad-spectrum antibiotics, which decimated the microbiota. Conversely, transplantation of the gut microbiota from these mice to wild-type mice induced weight gain and the metabolic syndrome phenotype. Our results indicate that the gut microbiota per se can subvert a genetically predetermined condition previously described as being protective towards obesity and insulin resistance into a phenotype associated with weight gain and its complications, such as glucose intolerance and diabetes.

executed by families of pattern-recognition molecules with a special role for Toll-like receptors (TLRs) [23,24]. Recent findings indicate that TLRs, which are up-regulated in the affected tissue of most inflammatory disorders, can mediate crosstalk between the immune systems and whole body metabolism [23]. It has been demonstrated that TLR4, a sensor for lipopolysaccharides on Gram-negative bacteria, is involved in the induction of proinflammatory cytokine expression in macrophages, adipocytes, and liver [13,25]. We and others have demonstrated that TLR4 genetically deficient mice or mice with an inactivating mutation for this receptor are substantially protected from obesity-induced insulin resistance [26,27]. Similarly, TLR2 genetically deficient mice are protected from high-fat-induced insulin resistance [28,29]. On the other hand, TLR5-deficient mice exhibit hyperphagia and develop hallmark features of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity [30], and these alterations are the consequence of alterations in the gut microbiota. It is important to emphasize that the studies that investigated TLR4- and TLR5-deficient mice were performed without germ-free conditions [26,27,30], suggesting that the microbiota have an important influence on TLR5-deficient mice phenotype, inducing obesity and insulin resistance; however, in the TLR4-deficient mice, the microbiota do not have a role in these phenomena because these animals are protected from diet-induced insulin resistance, independently of germ-free conditions [26,27]. Taken together, these findings suggest that the interaction of the innate immune system with gut microbiota may determine the insulin sensitivity of an animal and that TLRs may have different roles in this process. Since in most studies with TLR2-deficient mice the microbiota were not investigated, we cannot predict the influence of microbiota in the protection or in the development of insulin resistance in these mice. It is possible that the presence of a diverse gut microbiota could completely reverse the phenotype of an animal, inducing insulin resistance in an animal genetically determined to have increased insulin sensitivity, such as the TLR2 KO mice. The aim of the present study was to investigate the influence of gut microbiota in metabolic parameters, glucose tolerance, insulin sensitivity, and signaling of TLR2-deficient mice.

Results

Animal Characteristics

TLR2 KO mice did not present any difference in weight gain, compared with their controls up until 12 wk. However, after 12 wk, TLR2 KO mice were heavier than their controls ($p<0.05$; Figure 1A). No significant differences were observed with regard to food intake between the groups after either 8 or 16 wk (Figure 1B). The food intake was also normalized for body weight and no difference was observed between groups at 8 wk old (WT = 0.22±0.035 g/g animal/day; TLR2 KO = 0.21±0.021 g/g animal/day). However, after 16 wk, TLR2 KO mice presented increased epididymal fat weight (Figure 1C). After 12 wk, the amount of adipose tissue is visually increased in TLR2 KO mice (Figure 1D). It is interesting that TLR2 KO mice at 8 wk old had decreased glucose tolerance compared to their controls ($p<0.05$; Figure 1E), but no difference was observed in fasting serum insulin between the groups (Figure 1F). We next submitted these animals to a hyperinsulinemic euglycemic clamp to investigate insulin sensitivity; results showed that TLR2 KO mice presented a significant decrease in the rate of glucose uptake under high insulin stimulus (50% of control, $p<0.05$; Figure 1G), indicating a clear situation of insulin resistance.

We next analyzed the oxygen consumption from both groups and observed that TLR2 KO mice presented decreased oxygen consumption (Figure 1H), suggesting decreased energy expenditure when compared with their controls. However, the respiratory exchange ratio, approximately 0.85, was similar between the groups (Figure 1I). As the oxygen consumption was decreased in TLR2 KO mice, we evaluated a marker of thermogenesis in the brown adipose tissue of both groups. The expression of the thermogenesis-inducing protein, UCP1, was significantly decreased in TLR2 KO mice (Figure 1J), suggesting reduced energy expenditure in these animals, in accordance with the reduced oxygen consumption observed.

In order to characterize the gut microbiota of TLR2 KO mice, we pyrosequenced the 16S ribosomal RNA (rRNA) from the stools of these animals. TLR2 KO mice presented a different gut microbiota, compared with their controls. The major difference concerns the proportion of Firmicutes, which was approximately 47.92% in TLR2 KO mice, while the controls presented a proportion of 13.95%. Moreover, TLR2 KO mice presented 47.92% Bacteroidetes and 1.04% Proteobacteria, while their controls presented approximately 42.63% and 39.53%, respectively (Figure 2A,B). TLR2 KO mice presented other differences in regards to classes and families and these results are presented in the Supporting Information section (Figures S1 and S2).

However, it is important to notice that we have observed different proportions of these phyla between TLR2 KO mice and their controls in different ages of mice. From 4-wk-old to 1-y-old mice, we observed increased proportion of Firmicutes in TLR2 KO mice compared with the controls. We have also observed a tendency of decreasing the proportion of Bacteroidetes progressively as TLR2 KO mice get older (Figures S3, S4, S5).

Molecular Mechanisms of Insulin Resistance in TLR2 KO Mice

Next, we determined the serum concentration of IL-6 and TNF-α in both groups of animals and observed that TLR2 KO mice presented reduced levels of these cytokines compared with their

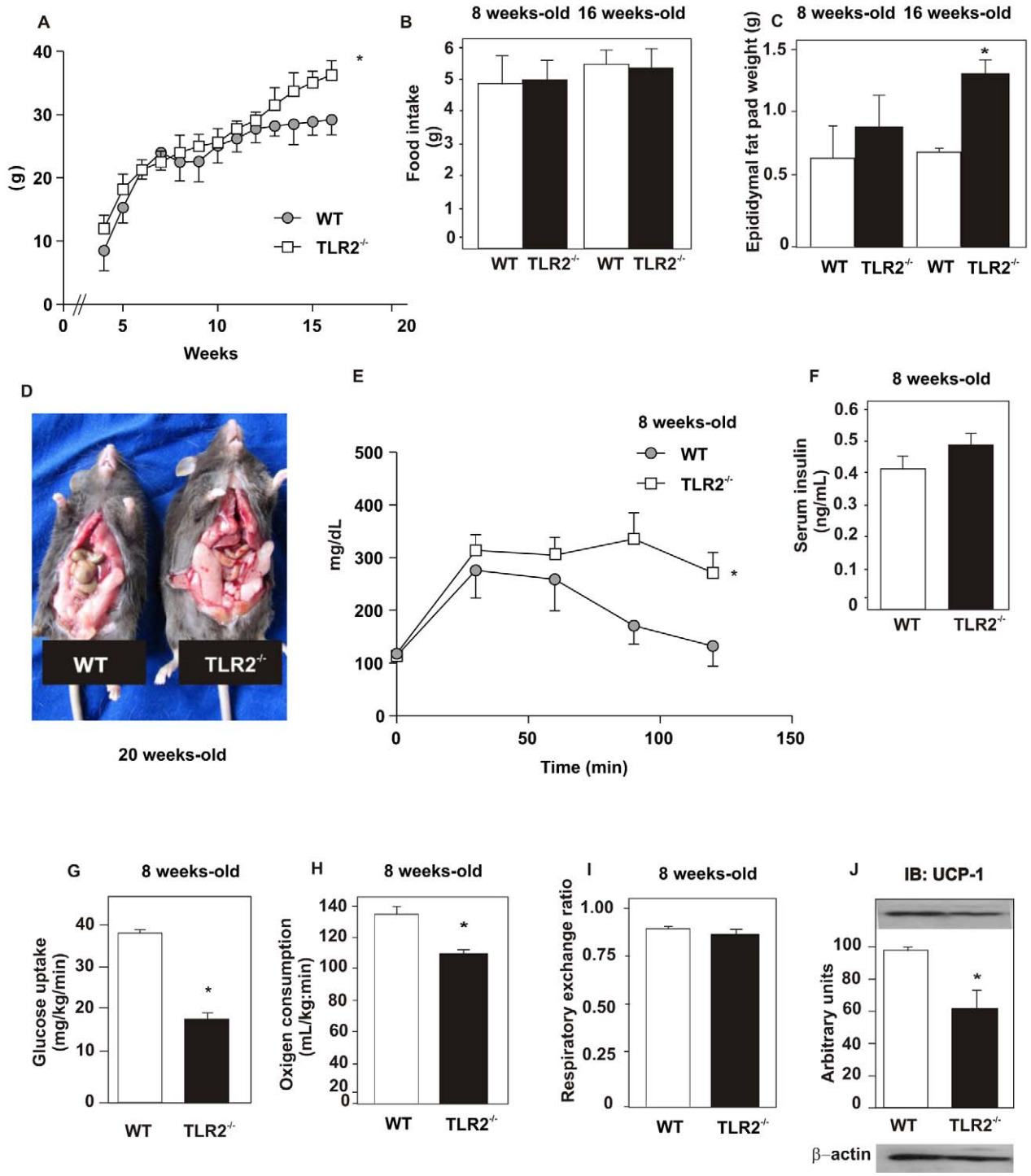

Figure 1. Metabolic parameters of TLR2 knockout (TLR2$^{-/-}$) and WT mice during 16 wk. (A) Weight gain after 16 wk. (B) Food intake after 8 and 16 wk. (C) Epididymal fat pad weight after 8 and 16 wk. (D) WT and TLR2−/− mice after 20 wk. (E) Glucose tolerance test. (F) Serum insulin concentration. (G) Glucose uptake obtained from euglycaemic hyperinsunaemic clamp. (H) Oxygen consumption and (I) respiratory exchange rate. (J) UCP-1 expression in the brown adipose tissue. Equal protein loading in the gel was confirmed by reblotting the membrane with an anti-β-actin antibody (J, lower panel). All evaluations were made with mice on standard chow. Data are presented as means ± S.E.M from six to eight mice per group from experiments that were repeated at least three times. *$p<0.05$ between TLR2−/− mice and their controls.
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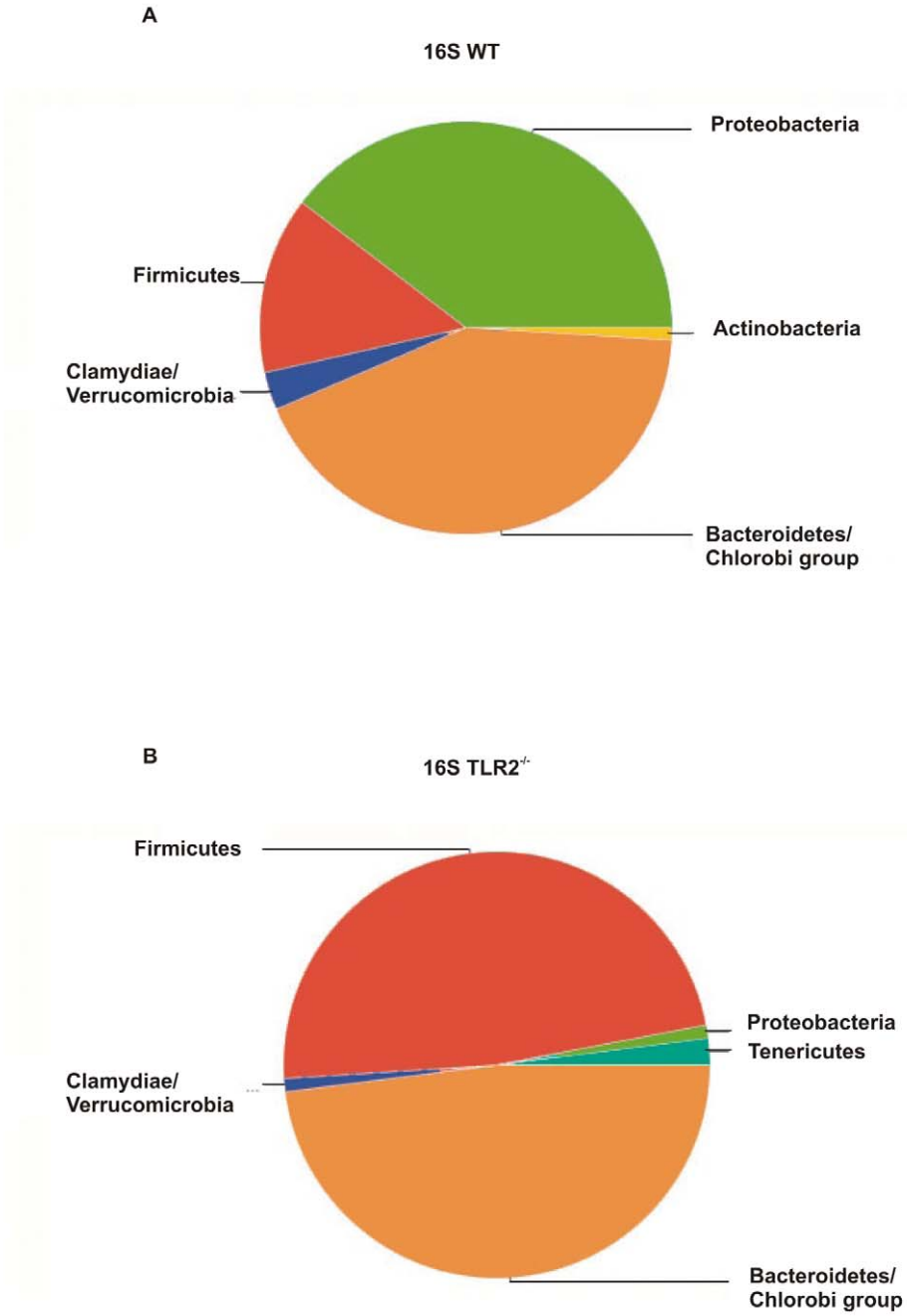

Figure 2. TLR2 KO mice exhibit taxonomical alterations in gut microbiota. Untreated WT (A) and TLR2 knockout (TLR2−/−) mice (B) stools were analyzed via 16S rRNA analysis. Data are presented from six to eight mice per group from experiments that were repeated at least three times. All evaluations were made with mice on standard chow.
doi:10.1371/journal.pbio.1001212.g002

controls (Figure 3A,B). We also investigated the serum concentrations of leptin, adiponectin, and LPS. No significant difference was observed between the groups with regard to leptin and adiponectin (Figure 3C,D). On the other hand, LPS serum concentration was increased in TLR2 KO mice (Figure 3E).

As TLR2 KO mice presented increased serum LPS levels, and this alteration was previously described in an animal model of obesity in which there was a reduced proportion of *Bifidobacterium* [31], we investigated the proportion of this group of bacteria. We observed that TLR2 KO mice presented a decrease in *Bifidobacterium* proportion compared with WT (Figure 3F).

In order to unravel the mechanism by which the insulin resistance occurs in the TLR2 KO mice, we studied important pathways involved in this phenomenon: phosphorylation of JNK, activation of ER stress, serine phosphorylation of the insulin receptor substrate (IRS)-1, and expression of IκB-α, which is involved in the inhibition of the IKK/NFκB pathway activation.

TLR2 KO mice presented increased phosphorylation of JNK in muscle, liver, and adipose tissue compared with controls (Figure 4A–C). Since the activation of ER stress leads to the phosphorylation of JNK, the increased phosphorylation of this protein in TLR2 KO mice could be due to this event. In fact, the

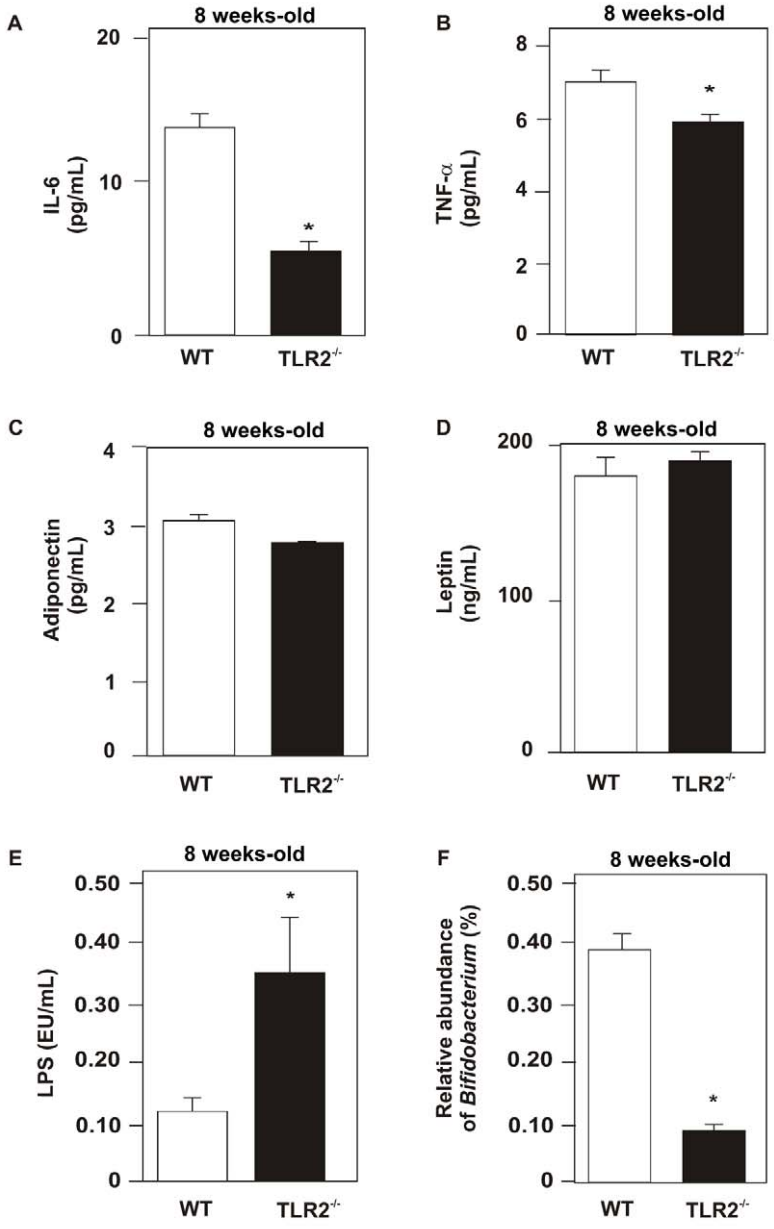

Figure 3. Measurements of cytokines, adipokines, and LPS. Serum concentration of IL-6 (A), TNF-α (B), adiponectin (C), leptin (D), and LPS (E). Proportion of *Bifidobacterium* was obtained by 16S rRNA analysis of stools (F). Data are presented as means ± S.E.M from six to eight mice per group from experiments that were repeated at least three times. * $p<0.05$ between TLR2 KO mice and their controls; all evaluations were made with mice on standard chow.
doi:10.1371/journal.pbio.1001212.g003

phosphorylation of PERK was increased in the liver and adipose tissue of the KO mice, suggesting increased ER stress activation at least in these two tissues (Figure 4D–F).

Next, we studied the inhibitory serine phosphorylation of IRS-1 in muscle, liver, and adipose tissue of TLR2 KO mice and observed that this phosphorylation was increased, compared with the controls, suggesting impairment of insulin signaling (Figure 4G–I). Since increased serum concentration of LPS, a TLR4 ligand, was observed in TLR2 KO mice, we investigated the activation of TLR4 in the muscle, liver, and adipose tissue of these mice. An increased activation of this receptor was observed in all tissues studied (Figure 4J–L), suggesting that, in the absence of TLR2, a compensatory action may lead to increased activation of TLR4, which may also contribute to the development of insulin resistance in TLR2 KO mice.

Then, we studied the activation of IKK/NFκB pathway, indirectly, by the expression of IκB-α. Curiously, the expression of IκB-α was increased in the muscle, liver, and white adipose tissue of TLR2 KO mice, compared with controls, suggesting a decreased activation of IKK/NFκB pathway (Figure 4M–O). In order to confirm this result, we studied the activation of NFκB and observed that this was decreased in all tissues studied from TLR2 KO mice (Figure 4P–R).

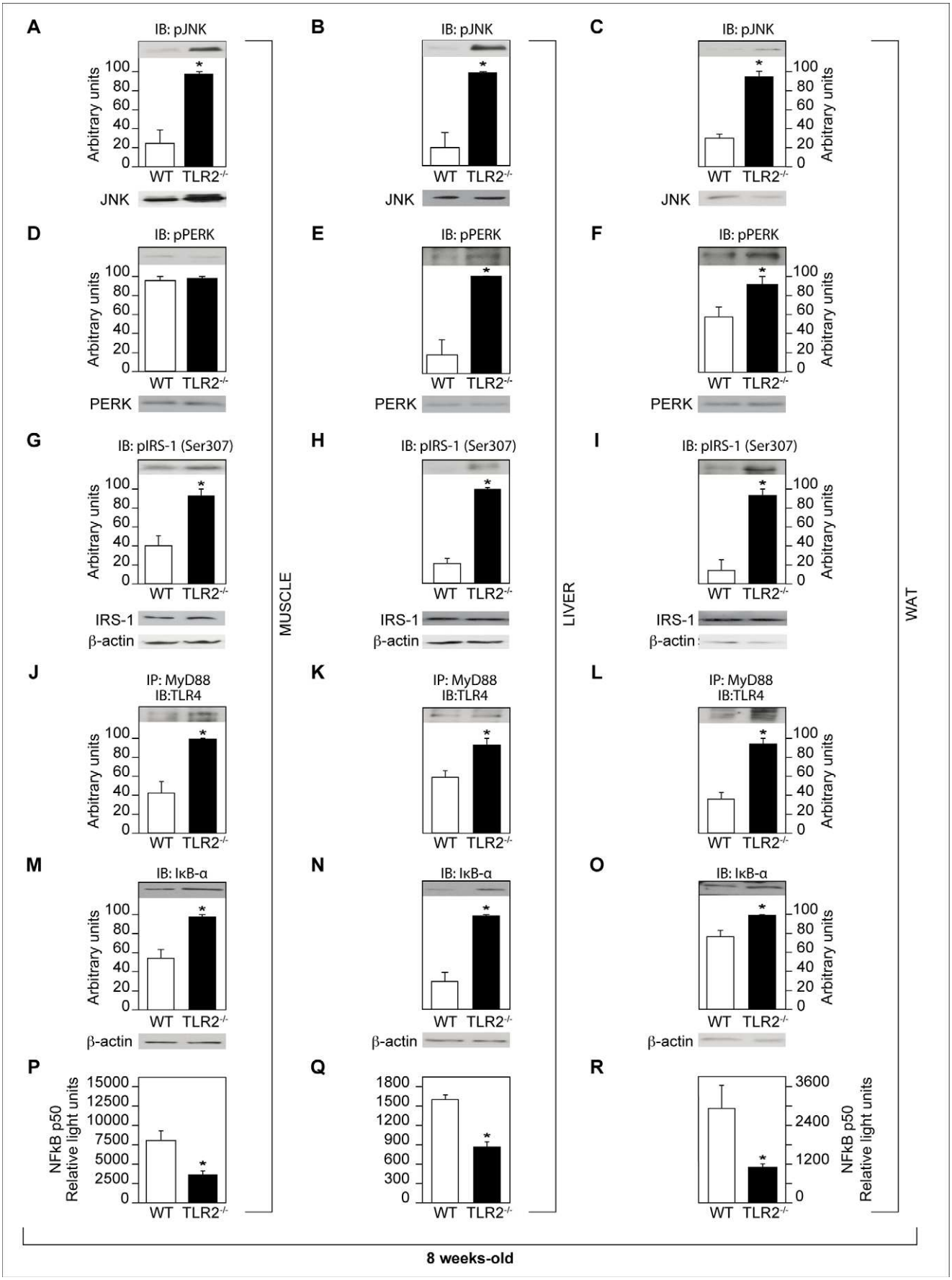
A
IB: pJNK
Arbitrary units
JNK
B
IB: pJNK
JNK
C
IB: pJNK
Arbitrary units
JNK
D
IB: pPERK
Arbitrary units
PERK
E
IB: pPERK
PERK
F
IB: pPERK
Arbitrary units
PERK
G
IB: pIRS-1 (Ser307)
Arbitrary units
IRS-1
β-actin
H
IB: pIRS-1 (Ser307)
IRS-1
β-actin
I
IB: pIRS-1 (Ser307)
Arbitrary units
IRS-1
β-actin
J
IP: MyD88
IB:TLR4
Arbitrary units
K
IP: MyD88
IB:TLR4
L
IP: MyD88
IB:TLR4
Arbitrary units
M
IB: IκB-α
Arbitrary units
β-actin
N
IB: IκB-α
β-actin
O
IB: IκB-α
Arbitrary units
β-actin
P
NFkB p50
Relative light units
Q
R
NFkB p50
Relative light units
WT
TLR2-/-
MUSCLE
LIVER
WAT
8 weeks-old

Figure 4. Evaluation of pathways involved in the impairment of insulin signaling. Phosphorylation of JNK in muscle (A), liver (B), and white adipose tissue (WAT) (C). Phosphorylation of PERK in muscle (D), liver (E), and WAT (F). Serine 307 phosphorylation of IRS-1 from muscle (G), liver (H), and WAT (I). Activation of TLR4 (studied by the immunoprecipitation of MyD88 and blotting with TLR4) in muscle (J), liver (K), and WAT (L). JNK, PERK, and IRS-1 protein expression in muscle, liver, and white adipose tissue (A–I, lower panels). Expression of IκB-α in muscle (M), liver (N), and WAT (O). Equal protein loading in the gel was confirmed by reblotting the membrane with an anti-β-actin antibody (M–O, lower panels). NFκB activation in muscle (P), liver (Q), and WAT (R). All evaluations were made with mice on standard chow. Data are presented as means ± S.E.M from six to eight mice per group from experiments that were repeated at least three times. * $p<0.05$ between TLR2−/− mice and their controls.
doi:10.1371/journal.pbio.1001212.g004

The insulin-induced tyrosine phosphorylation of the insulin receptor (IR) (Figure S6A–C) and of the insulin receptor substrate (IRS)-1 (Figure 5A–C), as well as the insulin-induced serine phosphorylation of AKT, was decreased in the muscle, liver, and adipose tissue of TLR2 KO mice (Figure 5D–F), compared with their controls, suggesting reduced insulin signaling in these tissues.

Other proteins that are important in the modulation of insulin action were also investigated. Our data showed that the phosphorylation of AMPK (Figure S6D–F) and the expression of PGC-1α (Figure S6G–I) were similar between controls and TLR2 KO mice in the three tissues investigated.

As an increased phosphorylation of JNK was observed in TLR2 KO mice, we prevented the activation of this protein with a pharmacological inhibitor, SP600125, by treating mice with daily i.p. injections for 5 d. Subsequently, we observed an increased glucose uptake in these animals, suggesting that the activation of JNK is indeed relevant to the development of insulin resistance (Figure 6A). We also observed increased insulin-induced serine

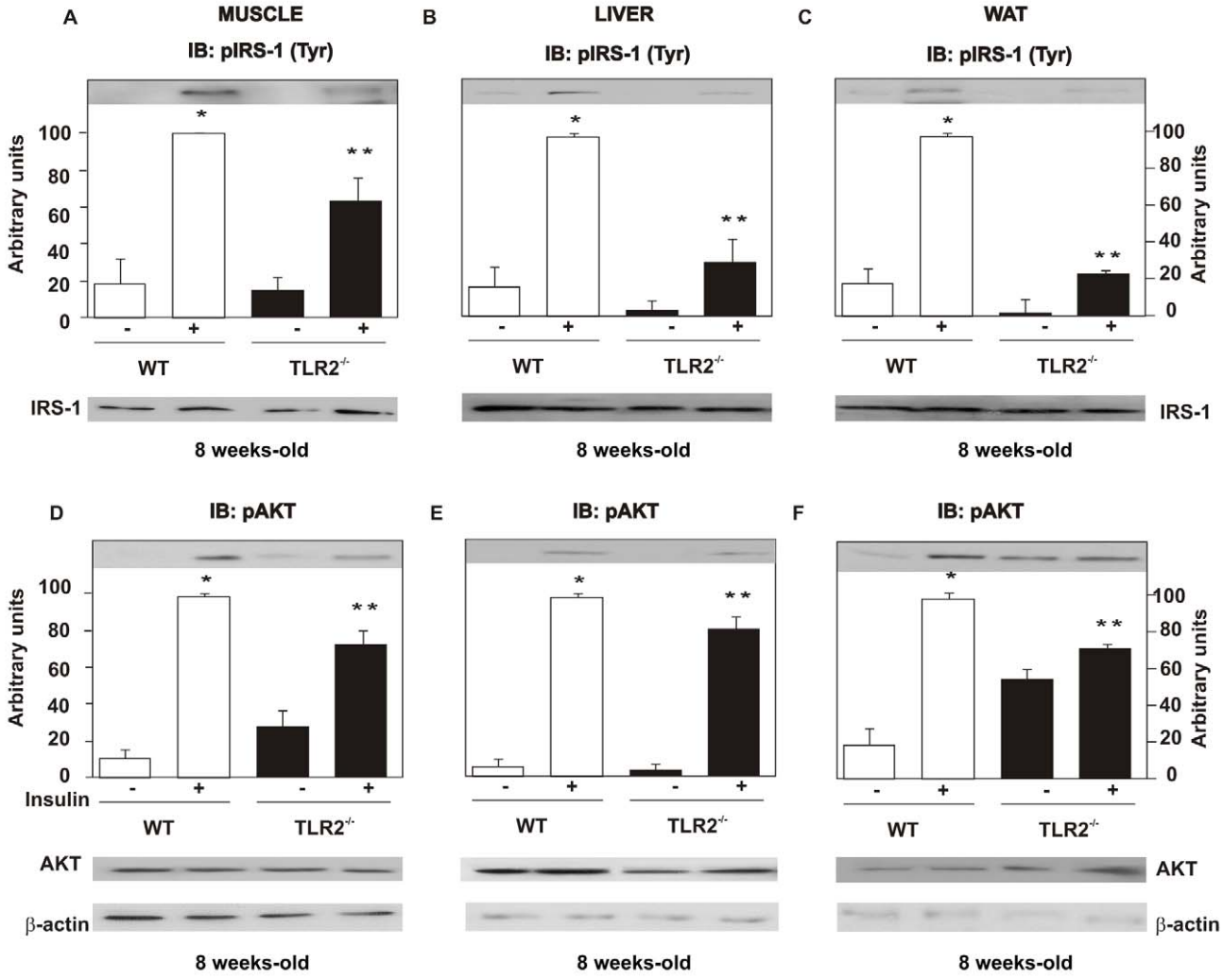

Figure 5. TLR2 knockout (TLR2−/−) mice present decreased insulin signaling. Tyrosine 941 phosphorylation of the insulin receptor substrate (IRS)-1 in muscle (A), liver (B), and WAT (C). Serine phosphorylation of AKT in muscle (D), liver (E), and WAT (F). IRS-1 and AKT protein expression in muscle, liver, and white adipose tissue (A–F, lower panels). Equal protein loading in the gel was confirmed by reblotting the membrane with an anti-β-actin antibody (lower panels). All evaluations were made with mice on standard chow. Data are presented as means ± S.E.M from six to eight mice per group from experiments that were repeated at least three times. *$p<0.05$ between WT with and without insulin stimulus; ** $p<0.05$ between TLR2−/− mice and their controls with insulin stimulus.
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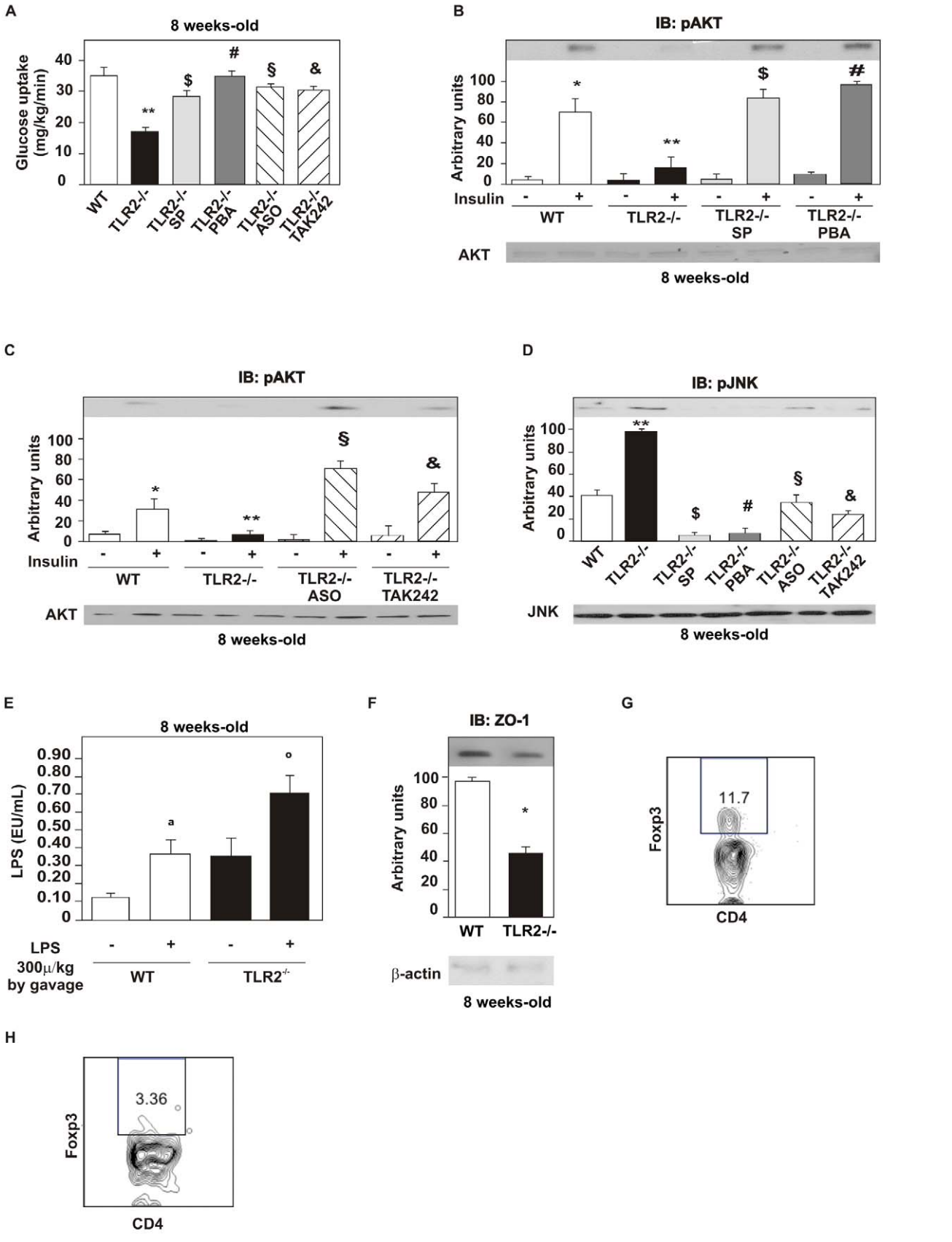
A
8 weeks-old
Glucose uptake (mg/kg/min)
WT
TLR2-/-
TLR2-/- SP
TLR2-/- PBA
TLR2-/- ASO
TLR2-/- TAK242
B
IB: pAKT
Arbitrary units
Insulin
WT
TLR2-/-
TLR2-/- SP
TLR2-/- PBA
AKT
8 weeks-old
C
IB: pAKT
Arbitrary units
Insulin
WT
TLR2-/-
TLR2-/- ASO
TLR2-/- TAK242
AKT
8 weeks-old
D
IB: pJNK
Arbitrary units
JNK
8 weeks-old
E
8 weeks-old
LPS (EU/mL)
LPS 300μ/kg by gavage
WT
TLR2-/-
F
IB: ZO-1
Arbitrary units
WT
TLR2-/-
β-actin
8 weeks-old
G
11.7
Foxp3
CD4
H
3.36
Foxp3
CD4

Figure 6. Insulin sensitivity and signaling after treatment with selective inhibitors. Glucose uptake obtained by the euglycaemic hyperinsulinaemic clamp from TLR2−/− mice treated or not with the drugs: SP600125 (SP), JNK inhibitor; 4-phenil butyric acid (PBA), endoplasmic reticulum stress inhibitor; TLR4 antisense oligonucleotide (ASO); (A) TAK-242, inhibitor of TLR4. (B) Serine phosphorylation of AKT after the treatment with SP600125 and PBA. (C) Serine phosphorylation of AKT after the treatment with TLR4 ASO and TAK-242. (D) Phosphorylation of JNK after the treatment with the drugs mentioned. Fasted TLR2 knockout mice and WT mice were gavaged by LPS (1.08, 10^{-8} g) diluted in water (100 μL) or without LPS. (E) Blood was collected from the cava vein 60 min after gavage and serum LPS was determined. (F) Zonula occludens (ZO)-1 expression in the ileum. (G) Frequency of CD4+Foxp3+ regulatory T cells in WT mice. (H) Frequency of CD4+Foxp3+ regulatory T cells in TLR2−/− mice. All evaluations were made with mice on standard chow. Data are presented as means ± S.E.M from six to eight mice per group from experiments that were repeated at least three times. * $p<0.05$ between WT mice with or without insulin stimulus; ** $p<0.05$ between WT and TLR2−/− mice with insulin stimulus ; $ $p<0.05$ between TLR2−/− mice with insulin stimulus, treated or not with SP; # $p<0.05$ between TLR2−/− mice with insulin stimulus, treated or not with PBA; § $p<0.05$ between TLR2−/− mice with insulin stimulus, treated or not with ASO; & $p<0.05$ between TLR2−/− mice with insulin stimulus, treated or not with TAK-242; [a] $p<0.05$ between WT mice with or without LPS stimulus; ° $p<0.05$ between WT and TLR2−/− mice with LPS stimulus.
doi:10.1371/journal.pbio.1001212.g006

phosphorylation of AKT in the liver (Figure 6B), muscle, and white adipose tissue (unpublished data) of TLR2 KO mice after this treatment, suggesting increased insulin signaling, as well, associated with a reduction in JNK phosphorylation in the liver of TLR2 KO mice (Figure 6D).

An increased activation of ER stress leads to the activation of JNK [32,33]. Therefore, we studied whether preventing the activation of this phenomenon could improve the insulin sensitivity and signaling. For this purpose, we treated mice with a pharmacological inhibitor of ER stress, 4-phenyl butyric acid (PBA), using i.p. daily injections for 10 d. This treatment was found to lead to an increased glucose uptake in TLR2 KO mice (Figure 6A) and increased insulin-induced serine phosphorylation of AKT in the liver (Figure 6B), muscle, and white adipose tissue (unpublished data), suggesting an improvement in the insulin signaling as well. After this treatment, we also investigated the phosphorylation of JNK and observed a reduction in the liver (Figure 6D) of TLR2 KO mice. Results suggest that both the activation of ER stress and the activation of JNK are important contributors to the development of the phenotype observed in TLR2 KO mice.

Since TLR4 was more activated in the tissues of TLR2 KO mice, possibly constituting one of the mechanisms responsible for the development of insulin resistance, we inhibited its expression using a TLR4 antisense oligonucleotide (ASO; with two daily i.p. injections) for 5 d. After TLR4 ASO treatment, TLR2 KO mice were found to present a significantly increased glucose uptake during the euglycemic hyperinsulinemic clamp compared with their controls (Figure 6A). The insulin signaling was also increased, with increased insulin-induced serine phosphorylation of AKT in the liver (Figure 6C), muscle, and white adipose tissue (unpublished data) of TLR2 KO mice. After this treatment, decreased phosphorylation of JNK was observed in the liver (Figure 6D) of TLR2 KO mice.

Using another method to inhibit TLR4 signaling, a pharmacological inhibitor of TLR4, TAK-242, was administered daily by gavage during 5 d and confirmed the results seen with the TLR4 ASO treatment. The insulin sensitivity was increased in TLR2 KO-treated animals (Figure 6A), and the insulin-induced serine phosphorylation of AKT was also increased in the liver (Figure 6C) of these animals, suggesting an improvement in insulin signaling. The phosphorylation of JNK was decreased in the liver (Figure 6D) of TLR2 KO treated mice.

As the serum LPS levels were increased in TLR2 KO mice, and the changes in microbiota may not account for this increase, we tested whether the LPS absorption was also increased in these animals. For this purpose, we administered LPS orally to TLR2 KO mice and wild type mice and determined the circulating LPS levels 1 h later. We observed that all animals presented increased serum LPS concentration after the LPS administration. However, TLR2 KO mice presented a higher increase in serum LPS concentration after the treatment, compared with the wild type mice (Figure 6E). As this result suggested that TLR2 KO mice presented increased gut permeability, we investigated the expression of an important tight-junction protein of the ileum of these mice, zonula occludens (ZO)-1, and observed that it was indeed decreased, compared with the control mice (Figure 6F). Reduction of ZO-1 expression in TLR2 KO mice was also observed in other parts of the small intestine and in the colon (unpublished data).

Previous data showed that TLR2 KO mice have a decreased number of regulatory T cells in the circulation compared with control mice [34]. This can have a role in the modulation of intestinal barrier and also in insulin resistance. We next investigated the frequency of Foxp3+ CD4+ T regulatory cells in mesenteric adipose tissue. We observed that the frequency of these cells was decreased in TLR2 KO mice (Figure 6H), compared with the wild type mice (Figure 6G).

Treatment of TLR2 KO Mice with Antibiotics Changes the Composition of Their Gut Microbiota and Improves Insulin Sensitivity

As the gut microbiota from TLR2 KO mice was shown to differ from that of controls, we treated both groups with a mixture of antibiotics (ampicillin, neomycin, and metronidazole) in their drinking water for 20 d. Moreover, we characterized the gut microbiota of TLR2 KO mice using culture-based microbial analysis of cecal contents after the antibiotics treatment and the results showed that aerobic bacteria were almost suppressed, while anaerobic bacteria decreased its abundance to 40% compared to the control group (Figure S7A).

After the treatment with the mixture of antibiotics, we also observed changes in the relative abundance of three phyla of bacteria. The abundance of Bacteroidetes was reduced from 47.92% to 19.78% and Firmicutes abundance decreased from 47.92% to 11.76% in the TLR2 KO mice, while Proteobacteria abundance increased from 1.04% to 67.38% in these mice (Figure S7B,C). TLR2 KO treated mice presented other differences in regards to classes and families and these results are presented in the Supporting Information section (Figures S8 and S9). When TLR2 KO mice were treated with metronidazole, neomycin, and ampicillin individually, and not as an antibiotics mixture, we observed that ampicillin was the most effective one to exterminate more bacteria diversity. When treated with metronidazole, TLR2 KO mice presented 46.51% of Proteobacteria, 10.69% of Firmicutes, and 42.32% of Bacteroidetes. When treated with neomycin, TLR2 KO mice presented 44.18% of Firmicutes and 55.81% of Bacteroidetes. When treated with ampicillin, almost 100% of the sequenced bacteria left corresponded to Proteobacteria (Figure S10A–C). Since the treatment with ampicillin or metronidazole normalized glucose tolerance in TLR2 KO mice,

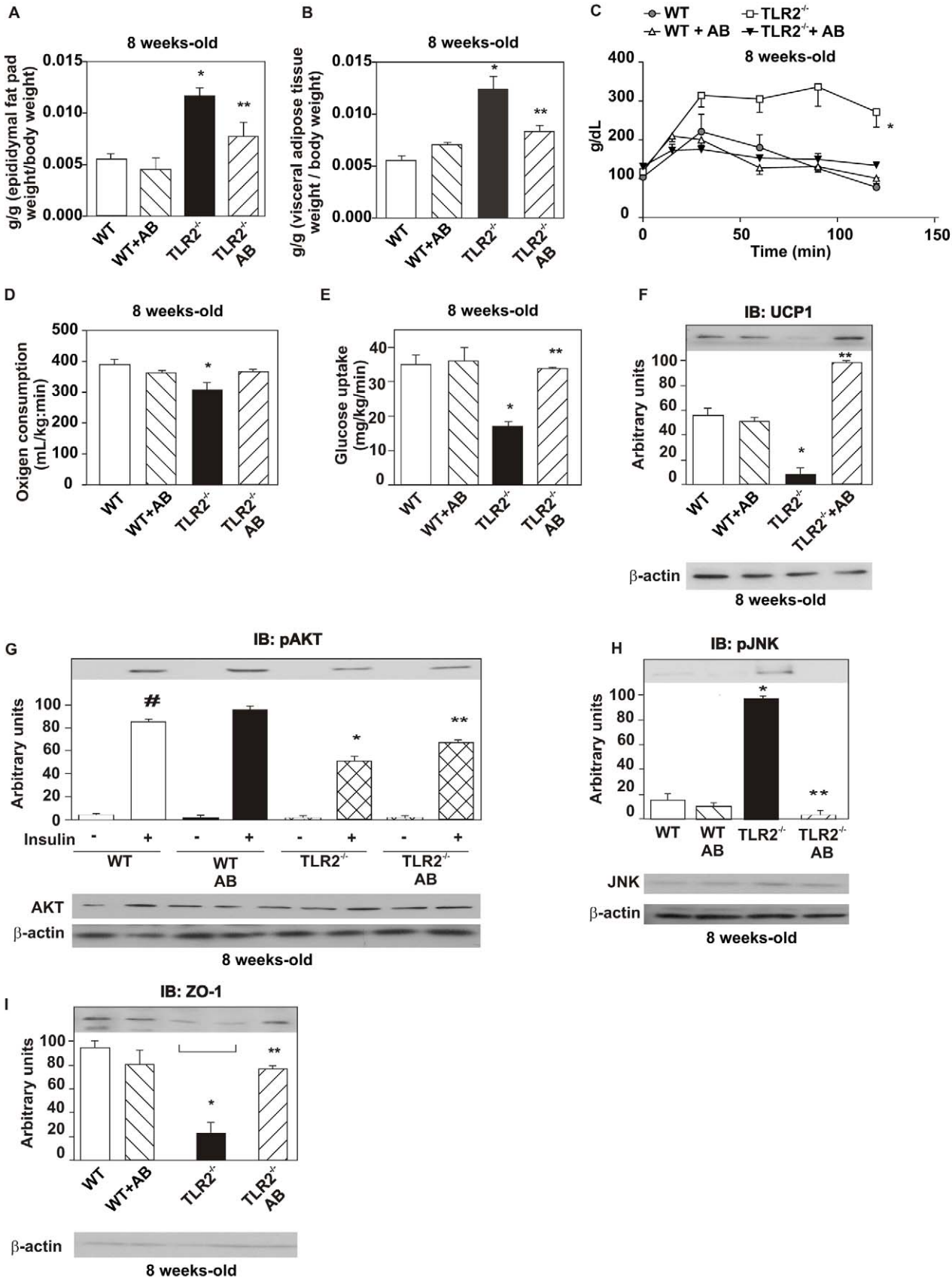

Figure 7. Alterations in the metabolic parameters and in insulin signaling and sensitivity after treatment with antibiotics. (A) Epididymal fat pad weight. (B) Visceral adipose tissue weight. (C) Glucose tolerance test. (D) Oxygen consumption. (E) Glucose uptake obtained by the euglycaemic hyperinsulinaemic clamp. (F) UCP-1 expression in the brown adipose tissue. (G) Serine phosphorylation of AKT after the treatment with

AB. (H) Phosphorylation of JNK after the treatment with AB. (I) Zonula occludens (ZO)-1 expression in the ileum. Equal protein loading in the gel was confirmed by reblotting the membrane with an anti-β-actin antibody (lower panels). Data are presented as means ± S.E.M from six to eight mice per group, from experiments that were repeated at least three times. All evaluations were made with mice on standard chow. # $p<0.05$ between WT mice with or without insulin stimulus; * $p<0.05$ between WT and TLR2−/− mice with insulin stimulus; ** $p<0.05$ between TLR2−/− and TLR2−/− treated with AB, with insulin stimulus.
doi:10.1371/journal.pbio.1001212.g007

and neomycin only mildly improved glucose tolerance in these mice (unpublished data), we can speculate that the changes in microbiota induced by ampicillin or metronidazole are more relevant than the changes induced by neomycin, although no specific genera of bacteria can be implicated in this response. However, in accordance with previous data on obese mice, the decrease in the proportion of the phylum Firmicutes, as observed in the groups that received ampicillin or metronidazole, correlates with the improvement in glucose tolerance.

TLR2 KO mice presented decreased epididymal fat pad and visceral adipose tissue weight after the treatment with antibiotics compared with non-treated TLR2 KO, while no difference was observed in the treated and non-treated control animals (Figure 7A,B). TLR2 KO mice also presented increased glucose tolerance (Figure 7C) and increased oxygen consumption (Figure 7D) after the treatment compared with non-treated TLR2 KO mice, but no significant difference was observed between treated and non-treated control mice. With regard to insulin sensitivity and signaling, we observed an improvement in insulin-induced glucose uptake, using the euglycemic hyperinsulinemic clamp, in TLR2 KO mice after antibiotics treatment (Figure 7E), with no difference in the treated and non-treated control mice. After the treatment, we also observed an increase in the UCP-1 expression in the brown adipose tissue of TLR2 KO mice, supporting the increased oxygen consumption observed in this condition (Figure 7F). We also observed an increased insulin-induced serine phosphorylation of AKT in the liver (Figure 7G), muscle, and white adipose tissue (unpublished data) of TLR2 KO mice after the treatment. Moreover, there was a decreased phosphorylation of JNK in the liver (Figure 7H), muscle, and white adipose tissue (unpublished data) of the knockout mice after the treatment. The antibiotics treatment also led to an increased expression of ZO-1 in TLR2 KO mice, with no difference in the treated and non-treated control mice (Figure 7I). These data suggest that, in TLR2 KO mice, the reduction in their gut microbiota associated with qualitative changes in composition, induced by antibiotics, was able to reverse the insulin resistance of these animals.

Effect of Microbiota Transplantation from TLR2 KO Mice to Control Mice on Weight Gain and Insulin Sensitivity

In order to investigate whether the gut microbiota was responsible for triggering all the alterations seen in TLR2 KO mice, we transplanted the cecal microbiota from TKR2 KO mice and from WT mice in 4-wk-old-*Bacillus*-associated WT mice, which contain few species of the genus *Bacillus*, without any other genera, as obtained by 16S rRNA pyrosequencing, in the following proportion: *Bacillus simplex* (0.68%), *Bacillus sp* (1.1%), *Bacillus sp* Kaza-34 (6.28%), and uncultured *Bacillus* (91.96%). There was a non-significant increase in the epididymal adipose tissue fat pad weight, in the total body weight gain, in the fasting blood glucose, and in the oxygen consumption in *Bacillus*-associated mice transplanted with WT microbiota (Figure 8A,C,D,G). However, in *Bacillus*-associated mice transplanted with TLR2 KO microbiota, we observed a marked increase in the epididymal fat pad and visceral adipose tissue weight (Figure 8A,B); in the body weight gain (Figure 8C), with a trend towards increased fasting blood glucose (Figure 8D), as well as a decrease in the glucose tolerance (Figure 8E,F); in the oxygen consumption (Figure 8G); and in the insulin sensitivity, obtained by euglycemic hyperinsulinemic clamp, compared with those transplanted with WT microbiota (Figure 8H) 8 wk after the transplantation ($p<0.05$). *Bacillus*-associated mice transplanted with WT microbiota also presented decreased insulin sensitivity compared with the non-transplanted mice ($p<0.05$). *Bacillus*-associated WT mice transplanted with TLR2 KO or with WT microbiota also showed decreased expression of UCP-1 in the brown adipose tissue compared with the non-transplanted mice. *Bacillus*-associated mice transplanted with TLR2 KO microbiota showed marked decrease in UCP-1 expression compared with those transplanted with WT microbiota (Figure 8I). Moreover, these animals had decreased insulin signaling, as seen by the reduction in serine phosphorylation of AKT in liver, compared to mice transplanted with WT microbiota (Figure 8J). In the mice transplanted with TLR2 KO microbiota, there was increased phosphorylation of JNK in liver (Figure 8K), muscle, and white adipose tissue (unpublished data) compared with the mice transplanted with WT microbiota. The experiments described above had also been performed in few germ-free mice, but with very similar results (unpublished data).

Eight weeks after the transplantation, the expression of ZO-1 was evaluated in the 12-wk-old mice. We observed that it was decreased in mice transplanted with TLR2 KO microbiota compared to those transplanted with WT microbiota (Figure 8L). The same data were observed in other parts of the small intestine and in the colon (unpublished data).

We also investigated the frequency of CD4+Foxp3+ regulatory T cells in these animals and observed that they were decreased in mesenteric adipose tissue in mice transplanted with TLR2 KO microbiota (Figure 8O) compared with the mice transplanted with WT microbiota (Figure 8N) and non-transplanted *Bacillus*-associated mice (Figure 8M).

In summary, as expected, the transplantation of a wild-type microbiota in *Bacillus*-associated mice resulted in a moderate increase in adipose visceral fat and in a mild decrease in glucose tolerance. However, the effect of the transplantation of TLR2 KO microbiota in *Bacillus*-associated mice induced marked changes, and clearly indicates deleterious effects of this TLR2 KO microbiota on body weight and glucose metabolism.

Effect of High-Fat Diet (HFD) on Weight Gain and Insulin Sensitivity in TLR2 KO Mice

Next, we investigated the effect of high-fat diet (HFD) on metabolic parameters of TLR2 KO mice. The results showed that at 8 wk old TLR2 KO mice on a HFD presented increased body weight (Figure 9A), similar food intake (WT = 7.3 g per day, TLR2 KO = 6.1 g per day; WT = 0.25±0.055 g/g animal/day; TLR2 KO = 0.19±0.046 g/g animal/day) (Figure 9B), increased epididymal fat weight (Figure 9C), reduced glucose tolerance (Figure 9D), increased fasting serum insulin (Figure 9E), and reduced glucose uptake (Figure 9F) compared to the controls. The oxygen consumption of both groups was compared and TLR2 KO mice were seen to present decreased oxygen consumption (Figure 9G), suggesting decreased energy expenditure compared to

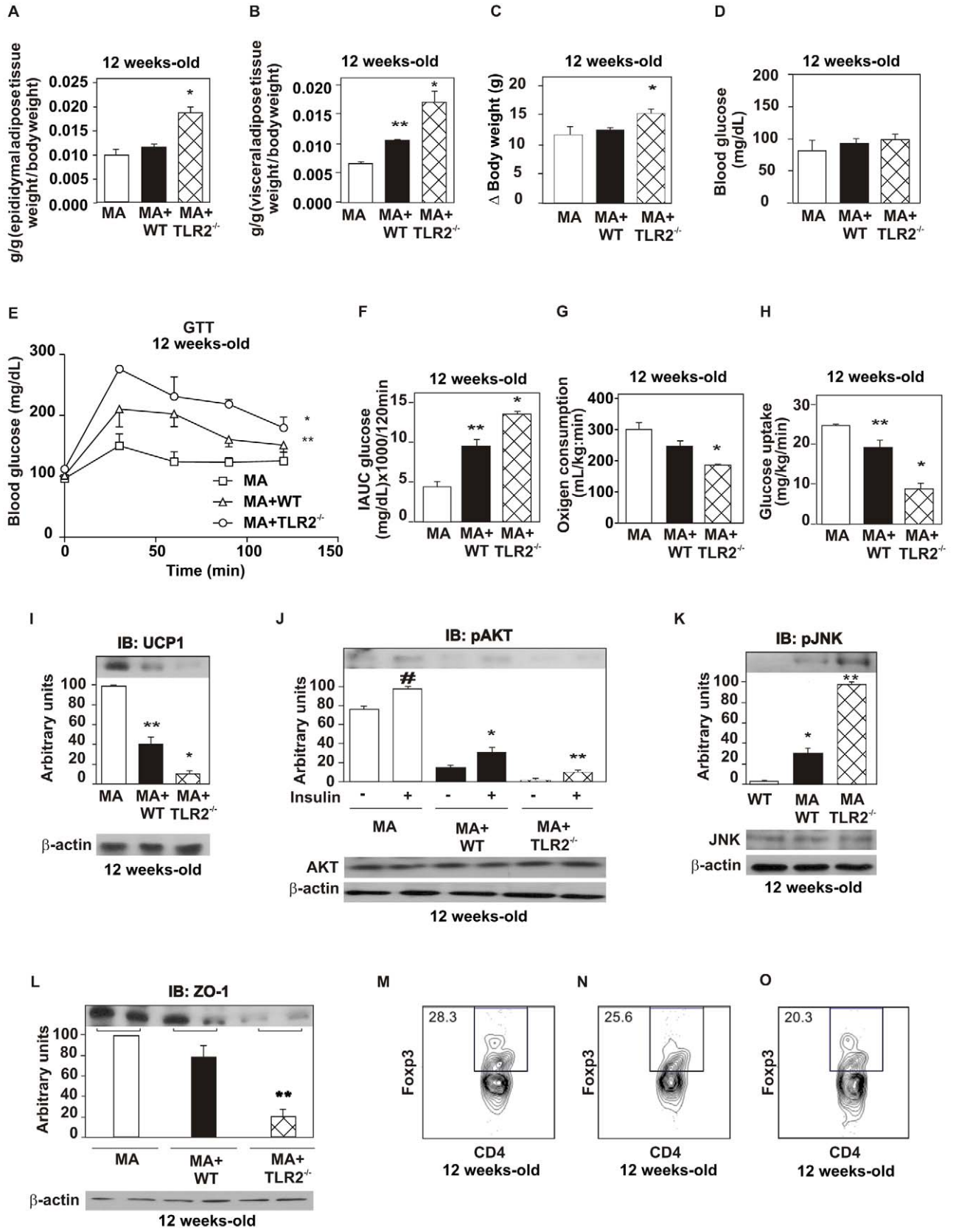

Figure 8. WT mice reproduce TLR2 knockout (TLR2−/−) mice after cecal microbiota transplantation. (A) Epididymal fat pad weight. (B) Visceral adipose tissue weight. (C) Weight gain of transplanted mice. (D) Serum glucose. (E) Glucose tolerance test. (F) Incremental area under curva (IAUC) obtained from the glucose tolerance test. (G) Oxygen consumption. (H) Glucose uptake obtained by the euglycaemic hyperinsulinaemic

clamp. (I) UCP-1 expression in the brown adipose tissue. (J) Serine phosphorylation of AKT after the treatment with AB. (K) Phosphorylation of JNK after the treatment with AB. AKT and JNK protein expression in the liver of transplanted mice (J, K, lower panels). (L) Zonula occludens (ZO)-1 expression in the ileum. (M) Frequency of CD4+Foxp3+ regulatory T cells in *Bacillus*-associated mice. (N) Frequency of CD4+Foxp3+ regulatory T cells in *Bacillus*-associated mice transplanted with WT microbiota. (O) Frequency of CD4+Foxp3+ regulatory T cells in *Bacillus*-associated mice transplanted with TLR2−/− microbiota. Equal protein loading in the gel was confirmed by reblotting the membrane with an anti-β-actin antibody (lower panels). Data are presented as means ± S.E.M from six to eight mice per group from experiments that were repeated at least three times. All evaluations were made with mice on standard chow. * $p<0.05$ between *Bacillus*-associated mice transplanted with TLR2−/− microbiota (MA+TLR2−/−) and those transplanted with WT microbiota (MA+WT); ** $p<0.05$ between *Bacillus*-associated mice transplanted with WT microbiota (MA+WT) and *Bacillus*-associated mice (MA); # $p<0.05$ between *Bacillus*-associated mice with or without insulin stimulus.
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the controls. However, the respiratory exchange ratio was similar in both groups, being around 0.75 (Figure 9H). In accordance with the reduced oxygen consumption observed, the expression of UCP1 was significantly decreased in TLR2 KO mice (Figure 9I). Similarly, insulin signaling was reduced in TLR2 KO mice fed on the HFD. The insulin-induced serine phosphorylation of AKT was reduced in the muscle, liver, and white adipose tissue of TLR2 KO mice, compared with controls (Figure 10A–C). Moreover, the phosphorylation of JNK was increased in all tissues studied of the TLR2 KO mice (Figure 10D–F), while the expression of IκB-α was increased (Figure 10G–I), suggesting that the IKK/NFκB pathway is decreased in TLR2 KO mice on a HFD, as observed for mice on a standard chow. These results suggest that the metabolic phenotype of the TLR2 KO mice characterized by insulin resistance is aggravated by HFD, which leads to the development of diabetes, as demonstrated by fasting blood glucose and glucose tolerance tests.

Discussion

It is now considered that environmental factors and host genetics interact to control the acquisition and stability of gut microbiota. In turn, environment, host genetics, and microbiota interact to maintain the homeostasis of gut, weight control, and insulin sensitivity [17]. Clearly, the modification of one or more of these three components may trigger the development of insulin resistance and obesity. The results of the present study demonstrated that TLR2 KO mice in conventionalized conditions in our breeding center have insulin resistance and glucose intolerance associated with alterations in the composition of the gut microbiota, which displayed an increase in the relative abundance of Firmicutes and Bacteroidetes and decreased relative abundance of Proteobacteria, compared to their controls. The insulin resistance of TLR2 KO mice was accompanied by a down-modulation of insulin-induced insulin signaling in the liver, muscle, and adipose tissue, associated with an increase in endoplasmic reticulum stress. These metabolic alterations were characterized in 8-wk-old TLR2 KO mice, when they had similar body weights to the control animals. As demonstrated in other animal models [35,36], this insulin resistance precedes the development of obesity, and an augmentation in body weight compared to controls is observed after the 12th wk of age.

However, previous studies [28,37] have reported that TLR2 KO mice present decreased body weight and adiposity, are protected against insulin resistance, and gain less weight on a HFD than control mice and are also protected against related comorbidities [38,39]. We believe that the main difference between these studies and our study may be related to gut microbiota. It should be taken into consideration that although the animals have the same genetic deficiency they were bred in different rooms and fed with food from different sources, which can certainly have a role in the establishment and maintenance of gut microbiota. Although in most of the previous studies the gut microbiota was not investigated, we can suggest that TLR2 deficiency associated with different environmental conditions can induce different phenotypes, probably induced by different microbiotas. Kellermayer et al. have shown that the proportion of Firmicutes found in TLR2 KO mice was lower than in WT, while the proportion of Bacteroidetes was increased [40]. In our study, we show that TLR2 KO mice present the opposite, with increased proportion of Firmicutes and decreased proportion of Bacteroidetes, compared with the WT. This way, it is possible that in the other published studies the proportions of this phyla might be different, compared with the proportions we have found, which might influence differently the phenotype observed. These results reinforce the importance of environment and of the innate immune system as key regulators of gut microbiota and suggest that a genetic condition, which by itself can prevent insulin resistance in some conditions, can also overcome the protective effect on insulin resistance in other environmental conditions inducing more weight gain, probably due to differences in the microbiota. In addition, these findings may help explain differences in the metabolic behavior of the same animal, when analyzed in distinct environments, and can contribute to explaining differences in metabolic behavior between animals with the same background or with the same genetic alteration.

The mechanisms by which the TLR2 KO mice presented insulin resistance and, later, obesity were also investigated. The gut microbiota of the TLR2 KO animal have some similarities to those found in obese animals and humans, with an increase in Firmicutes [41,42]. This type of microbiota is usually associated with an increased capacity for energy harvesting from the diet [19]. This might contribute to explaining the obesity observed, but does not explain why these animals are clearly insulin resistant many weeks before they start to gain more weight than their controls. In addition, it was demonstrated that germ-free (that gain less weight on HFD) and conventionalized mice have similar energy contents in their feces, suggesting that other mechanisms may have an important role in gut microbiota-induced insulin resistance and obesity [43]. Additionally some studies suggest that the gut microbiota can contribute to obesity by inducing a reduction in fat oxidation and an increase in fat storage [43], associated with a relative reduction in the expression of PGC1 alpha and in AMPK phosphorylation. This mechanism is less probable in our animal model, because the RQ of TLR2 KO mice was identical to that of control mice, showing that they were oxidizing fat in the same proportion of controls, and also the tissue levels of PGC1alpha and also the phosphorylation of AMPK were similar in liver and muscle of controls and TLR2 KO mice.

Another possible mechanism that could induce insulin resistance in obesity is the increased level of LPS, which is observed in HFD mice [11,44]. Notably, although TLR2 KO mice were fed on standard rodent chow, they presented higher circulating levels of LPS. Since the microbiota of these mice had a predominance of Firmicutes, which are gram-positive, and do not have LPS in the outer membrane, the increase of LPS circulating levels is certainly not the consequence of a microbiota that produces more LPS. However, the microbiota observed in obesity and also in TLR2

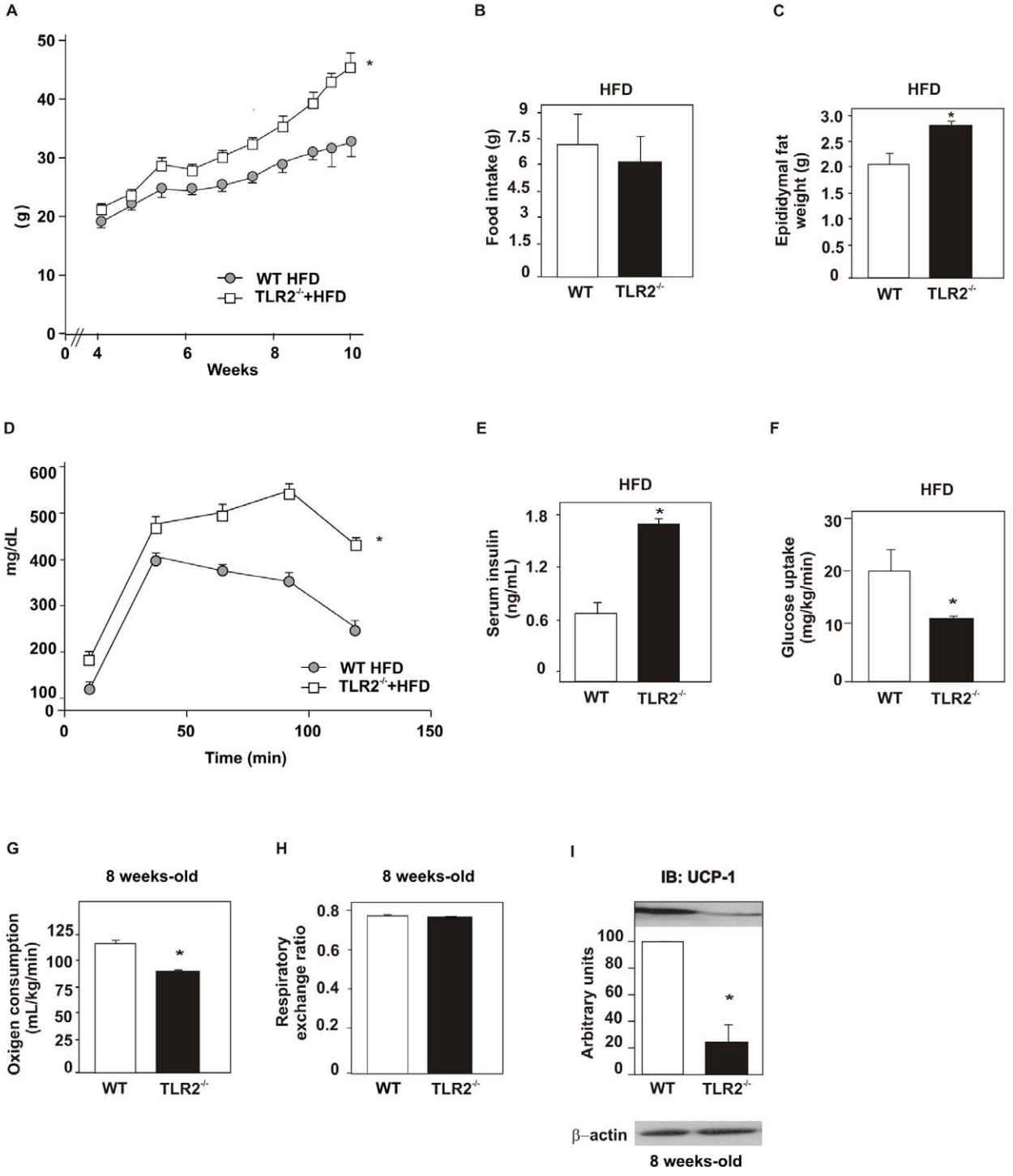

Figure 9. Metabolic parameters of TLR2 KO (TLR2−/−) mice fed a high-fat diet. (A) Weight gain after 10 wk of high-fat diet (HFD). (B) Food intake. (C) Epididymal fat pad weight. (D) Glucose tolerance test. (E) Serum insulin concentration. (F) Glucose uptake obtained from the euglycaemic hyperinsulinaemic clamp. (G) Oxygen consumption and (H) respiratory exchange rate. (I) UCP-1 expression in the brown adipose tissue. Data are presented as means ± S.E.M from six to eight mice per group from experiments that were repeated at least three times. All evaluations were made with mice on standard chow. * $p<0.05$ between TLR2−/− mice and their controls.
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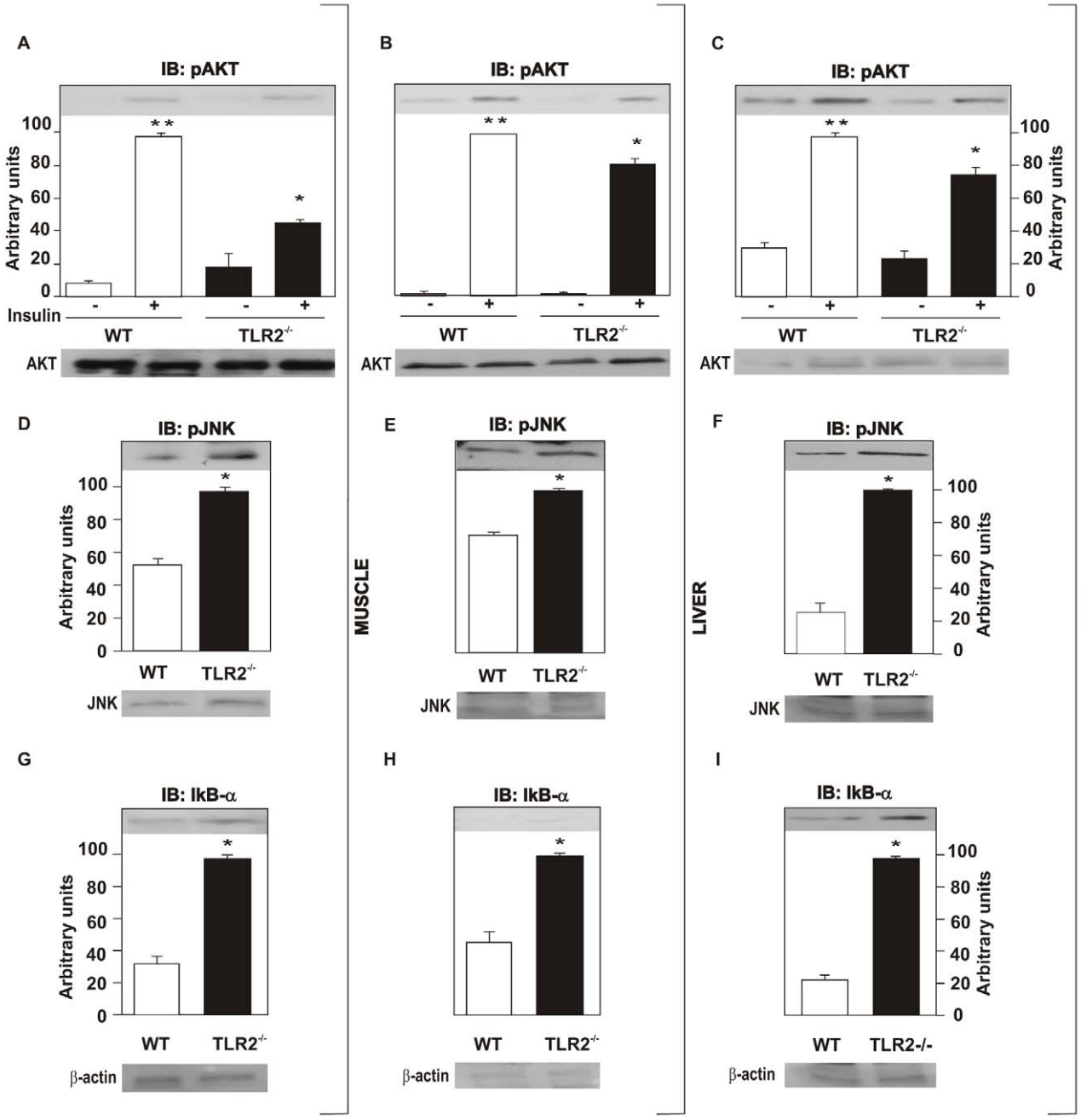

Figure 10. Insulin signaling is impaired in TLR2 knockout (TLR2−/−) mice fed on a high-fat diet. Phosphorylation of AKT in muscle (A), liver (B), and white adipose tissue (WAT) (C). AKT protein expression in muscle, liver, and WAT (A–C, lower panels). Phosphorylation of JNK in muscle (D), liver (E), and WAT (F). JNK protein expression in muscle, liver, and WAT (D–F, lower panels). IκB-α expression in muscle (G), liver (H), and WAT (I). Equal protein loading in the gel was confirmed by reblotting the membrane with an anti-β-actin antibody (lower panels). All evaluations were made with mice on standard chow. Data are presented as means ± S.E.M from six to eight mice per group from experiments that were repeated at least three times. * $p<0.05$ between TLR2−/− mice and their controls, with insulin stimulus; ** $p<0.05$ between WT with and without insulin stimulus.
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KO mice may increase gut permeability and LPS absorption [45–47]. Importantly, as observed in obese animals, which present a significant reduction in Bifidobacteria [48,49], in the microbiota of lean TLR2 KO mice this genera was reduced compared with the controls. In this regard, the supplementation of Bifidobacteria has been linked to an improvement in the gut barrier function and to reduced levels of LPS [31,50,51]. In order to prove that the increased circulating LPS levels of TLR2 KO mice were related to gut permeability, we administered LPS orally to these mice and observed that, in addition to higher basal LPS levels, these animals also showed a higher peak of LPS 1 h after oral gavage of this lipopolysaccharide.

Previous data showed that TLR2 regulates tight junction (TJ)-associated intestinal epithelial barrier integrity and that TLR2 deficiency predisposes to alterations of TJ-modulated barrier function leading to perpetuation of mucosal inflammation [52,53].

In this regard, our data also demonstrated that, in TLR2 KO mice, there is a reduction in ZO-1 in the small intestine and in the colon, reinforcing that there are alterations in epithelial integrity and gut permeability in these mice. Taken together, these results suggest that the interactions between the predisposition of TLR2 KO mice to alterations in barrier function and the microbiota may have an important role in the increased circulating LPS levels observed in these mice.

In accordance with alterations in gut permeability, Kellermayer et al. recently investigated the epigenomic and metagenomic consequences of *Tlr2* deficiency in the colonic mucosa of mice in order to understand the biological pathways that shape the interface between the gut microbiota and the mammalian host. The results showed epigenomic and transcriptomic modifications associated with alterations in mucosal microbial composition and the abundance of many bacterial species were found to differ between WT and TLR2 KO animals. The expression of genes involved in the immune system was modified in the colonic mucosa of TLR2 KO mice, which correlated with DNA methylation changes. This pioneer study demonstrates that significant microbiota shifts associate with epithelial epigenetic changes influenced by the host genome [54].

In order to confirm that gut microbiota was inducing insulin resistance in TLR2 KO mice, we treated these mice with antibiotics for 15 d and showed that this treatment dramatically reduced the gut microbiota and also changed its composition. In parallel, there was an improvement in insulin action, characterized by an increased glucose infusion rate during the glucose clamp, and also an improvement in insulin signaling in the liver, muscle, and adipose tissue. In the TLR2 KO mice treated with antibiotics, we also observed a marked reduction in LPS levels. When we performed gut microbiota transplantation of TLR2 KO mice to *Bacillus*-associated WT mice, which are colonized only by the genus *Bacillus* and are capable of receiving a different microbiota from other mice, the complex composition of the transferred organism was preserved. The transplanted TLR2 KO mice microbiota conferred more weight gain, glucose intolerance, and reduced insulin sensitivity and signaling, associated with increased LPS circulating levels. These data reinforce the hypothesis that the TLR2 KO mice microbiota are able to induce changes in the gut permeability, in turn increasing serum LPS levels, associated with insulin resistance.

The increase in LPS may induce insulin resistance by counteracting insulin signaling, as previously demonstrated [11,55,56]. However, the insulin resistance observed in TLR2 KO mice has unique characteristics. There was activation of TLR4 in the liver, muscle, and adipose tissue, associated with ER stress and JNK activation, but no activation of the IKKβ-IκB-NFκB pathway. It was previously described that there is cooperation between TLR4 and TLR2 signaling. This cooperation is evident when LPS is injected in TLR2 KO mice. After the first bolus of LPS, TLR2 KO mice show a robust signal for genes encoding innate immune proteins in the brain. However, the second LPS infusion failed to trigger TNFalpha in TLR2 KO mice. These results indicate that TLR2 is involved in the second wave of TNFalpha expression after LPS and that there is an elegant cooperation between TLR2 and TLR4 [57]. Our results extended these data by showing that the chronic elevation in LPS levels in TLR2 KO mice was not able to increase IKK/IkB/NF-kB pathway and TNFalpha and IL-6 production, but induced an increase in JNK activation in liver, muscle, and adipose tissue of these mice. These data suggest that chronic activation of TLR4 by low doses of LPS is sufficient to increase JNK activation, but the activation of IKK/IkB/NF-kB pathway may also depend on the cooperation between TLR2 and TLR4.

The absence of activation of the NFκB pathway and the reduced levels of TNFα and IL-6 make the insulin resistance of TLR2 KO mice different from that observed in DIO mice or in ob/ob mice. We can, thus, suggest that the increase in LPS circulating levels caused activation of TLR4, induced ER stress and JNK activation accompanied by increased IRS-1 serine 307 phosphorylation in the liver, muscle, and adipose tissue, leading to a reduction in insulin sensitivity and signaling and conferring the phenotype observed in the TLR2 KO mice. Phosphorylation of IRS-1 on serine residues interferes with the subsequent insulin-stimulated tyrosine phosphorylation of IRS-1 by IR [58] and IRS-1 can also mediate inhibition of the insulin receptor tyrosine kinase activity [55], and also with downstream signaling as Akt phosphorylation. This insulin signaling pathway is crucial for the metabolic effects of insulin on glucose metabolism [59]. The pharmacological or genetic blockage of TLR4, of ER stress, or of JNK improved action and signaling of insulin in TLR2 KO mice, confirming that this sequence of events has an important role in the insulin resistance of these animals.

Regulatory T cells, a small subset of T lymphocytes, are thought to be one of the body's most important defenses against inappropriate immune responses [60,61] and can influence the activities of cells of the innate immune system [62–64]. Previous data showed that regulatory T cells were highly enriched in the abdominal fat of control mice and reduced at this site in animal models of obesity. This reduction in obesity of regulatory T cells influenced the inflammatory state of adipose tissue and certainly contributes to insulin resistance. Our data showing that in TLR2 KO mice there is a reduction in regulatory T cell in visceral adipose tissue may suggest that this modulation may also contribute to the insulin resistance observed in these animals.

The development of obesity and insulin resistance in humans is thought to be promoted by a HFD. Feeding TLR2 KO mice with a HFD for 8 wk caused a marked increase in body weight and in fasting plasma glucose, with levels of over 400 mg/dl at 2 h during the glucose tolerance test, demonstrating that these animals developed not only a more severe form of insulin resistance but also diabetes. The alterations in insulin signaling in tissues also showed a marked down-regulation, in parallel with a higher activation of JNK compared to their controls on HFD. Interestingly, the absence of activation of the IKKβ-IκB-NFκB pathway, described in TLR2 KO mice on standard rodent chow, was also observed when these mice were on HFD. These results demonstrate that the insulin resistance, and later the increase in body weight observed in TLR2 KO mice, is exacerbated by HFD.

A recent report demonstrated that genetically deficient TLR5 mice exhibit hyperphagia, hyperlipidemia, insulin resistance, and increased adiposity [30]. These metabolic alterations correlated with changes in the composition of the gut microbiota. Our model, although showing similar features, presented different aspects that may suggest that different mechanisms may be operating in TLR5 or TLR2 KO mice. First, TLR2 KO mice did not present hyperphagia, and the difference in body weight starts only when these animals are 16 wk old. In the TLR5 KO mice, the insulin resistance is not dependent on TLR4, but in TLR2 KO mice there is an increase in circulating LPS and activation of TLR4. It is possible that these differences not only represent differences in genetic defects but also differences in gut microbiota between these mice.

In conclusion, we may suggest that the loss of TLR2 in conventionalized mice results in a reminiscent phenotype of metabolic syndrome, characterized by a clear difference in the gut

microbiota, which induces insulin resistance, subclinical inflammation associated with ER stress, glucose intolerance, and later obesity, which is reproduced in WT by microbiota transplantation and can be reversed using antibiotics. Our results emphasize the role of microbiota in the complex network of molecular and cellular interactions that bridge genotype to phenotype and have potential implications for a wide array of common human disorders involving obesity, diabetes, and even other immunological disorders.

Materials and Methods

Materials

Human recombinant insulin was from Eli Lilly (Indianapolis, Indiana, USA). Reagents for SDS-PAGE and immunoblotting were from Bio-Rad. HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, and bovine serum albumin (fraction V) were from Sigma. Protein A-Sepharose 6MB was from GE Healthcare, and nitrocellulose paper (BA85, 0.2 μm) was from Amersham Biosciences. The reagents for the chemiluminescence labeling of proteins in blots were from Amersham Biosciences. Sense and antisense oligonucleotides specific for TLR4 (sense, 5′-C TGA AAA AGC ATT CCC ACC T-3′ and antisense, 5′-A GGT GGG AAT GCT TTT TCA G-3′) were produced by Invitrogen Corp. (Carlsbad, CA). Antibodies against β-actin (mouse monoclonal, sc-8432), TLR4 (rabbit polyclonal, sc-30002), phospho [Ser307]-IRS-1 (rabbit polyclonal, sc-33956), phospho [Tyr941] (goat polyclonal, sc-17199), IRS-1 (rabbit polyclonal, sc-559), phospho [Ser 473]-AKT (rabbit polyclonal, sc-33437), AKT1 (goat polyclonal, sc-1618), phospho [Thr 981]-PERK (rabbit polyclonal, sc-32577), PERK (goat polyclonal, sc-9477), phospho-JNK (mouse monoclonal, sc-6254), JNK1 (mouse monoclonal, sc-1648), phospho[Tyr1162/1163]-Insulin Receptor (rabbit polyclonal, sc-25103), Insulin Receptor β (goat polyclonal, sc-31369), UCP1 (goat polyclonal sc-6529), and MyD88 (goat polyclonal, sc-8197) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against ZO-1 was from Abcam (AB96594) (Cambridge, MA). Antibodies against phospho [Thr172]-AMPKα (rabbit polyclonal, #2531), AMPKα (rabbit polyclonal, #2532), and IκB-α (rabbit polyclonal, #9242) were from Cell Signaling Technology (Beverly, Massachusetts, USA).

Mice

TLR2-deficient mice, also called TLR2 knockout (KO) mice, were obtained by Dr. Akira [65] and were kindly provided by Dr. Ricardo Gazzinelli [66] and maintained on a C57BL/6J genetic background. Studies were carried out using male TLR2 KO mice that were age matched with C57BL/6J and obtained from the University of Campinas Breeding Center. C57BL/6J and the TLR2 KO mice have the same origin and have been raised in the same institution (UNICAMP) and in the same room, at University of Campinas Breeding Center. The C57BL/6J strain was generated by backcrossing mice carrying the TLR2 KO mutation 10 times to C57BL/6J inbred mice [67]. TLR2-deficient mice are viable and fertile. The control and the knockout mice used for the experiments were littermates, obtained from a heterozygote × heterozygote cross, from the same mother, from the same cage, in order to have standard conditions for all animals. The investigation was approved by the ethics committee and followed the university guidelines for the use of animals in experimental studies, and experiments conform to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication no. 85–23 revised 1996). The animals were maintained on 12 h/12 h artificial light-dark cycles and housed in individual cages. Mice were randomly divided into two groups: control, fed on standard rodent chow (3.948 kcal/Kg^{-1}), and HFD, fed on a rich-fat chow (5.358 kcal/Kg^{-1}) *ad libitum* for 16 wk. The mice were bred under specific pathogen-free conditions at the Central Breeding Center of the University of Campinas.

Serum Analysis

Mice were fasted for 5 h, at which time blood was collected by the retrobulbar intraorbital capillary plexus. Hemolysis-free serum was generated by the centrifugation of blood using serum separator tubes (Becton Dickinson, Franklin Lakes, New Jersey). Serum insulin, cytokines, leptin, and adiponectin were analyzed by ELISA kits purchased from Linco Research Inc (St. Charles, Missouri).

Determination of NF-κB Activation

NF-κB p50 activation was determined in nuclear extracts from muscle and adipose tissue by ELISA (89858; Pierce Biotechnology), according to the recommendations of the manufacturer.

LPS Serum Determination

Serum LPS concentration was determined using a kit based on a Limulus amebocyte extract (LAL kit endpoint-QCL1000; Cambrex BioScience, Walkersville, Maryland), where samples were diluted 1/40 to 1/100 and heated for 10 min at 70°C. Internal control of recovery calculation was included in the assessment.

Glucose Tolerance Test

After 6 h fasting, mice were anesthetized by an i.p. injection of sodium amobarbital (15 mg/kg body weight), and the experiments were initiated after the loss of corneal and pedal reflexes. After collection of an unchallenged sample (time 0), a solution of 20% glucose (2.0 g/kg body weight) was administered into the peritoneal cavity. Blood samples were collected from the tail at 30, 60, 90, and 120 min for determination of glucose and insulin concentrations [68].

Euglycaemic-Hyperinsulinaemic Clamp

After a 6-h fast, a prime continuous (3.0 $mU \cdot kg^{-1} \cdot min^{-1}$) infusion of regular insulin was administered in the groups of mice for 2 h from time 0, to raise plasma insulin and maintain it at a steady-state plateau (90–120 min). A variable glucose infusion (10%) was started 5 min after the beginning of the experiment and was corrected, if necessary, to maintain euglycaemia between 5 and 6.1 mmol/l [69]. Blood samples for determination of plasma glucose were obtained at 5-min intervals throughout the study.

Oxygen Consumption/Carbon Dioxide Production and Respiratory Exchange Ratio Determination

Oxygen consumption/carbon dioxide production and respiratory exchange ratio (RER) were measured in fed animals through an indirect open circuit calorimeter (Oxymax Deluxe System; Columbus Instruments, Columbus, Ohio), as described previously [70].

Measurement of Food Intake

Standard chow or HFD was given and food intake was determined by measuring the difference between the weight of chow given and the weight of chow at the end of a 24-h period. This procedure was performed during 5 d, with 8-wk-old mice,

using metabolic cages for a single mouse (Tecniplast, Italy), obtaining an average of food intake per cage per day. This average was also normalized for body weight.

4-Phenyl Butyric Acid (PBA) Treatment

PBA is a chemical chaperone and evidence suggests that it relieves endoplasmic reticulum stress [71]. For acclimation, mice received 100 µl phosphate buffered saline (PBS) twice daily (8 a.m. and 6 p.m.), by gavage, for 3 d. Following the acclimation period, PBA was administered twice daily in two divided doses (500 mg/kg at 8 a.m. and at 6 p.m., total 1 g/kg/day) by gavage for 10 d. Control groups received the same volume of vehicle instead of PBA at the same treatment points [33].

SP600125 Treatment

SP600125, a potent and selective inhibitor of JNK, was dissolved in a 7% (in PBS) Solutol HS-15 solution and administered intraperitoneally (30 mg/kg/day) for 5 d [72].

TLR4 Inhibition

In order to inhibit the expression of TLR4, two methods were used: pharmacological inhibition, using 2.4 mg/kg/day ethyl(6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242) (synthesized at the Chemistry Institute of the University of Campinas) [73], administered daily by gavage during 5 d, and 4 nmol TLR2 antisense oligonucleotide (ASO) inhibition, composed by 5′-AGGTGGGAATGCTTTTTCAG-3′ (sense) and 5′-CTGAAAAAGCATTCCCACCT-3′ (antisense), administered by two daily i.p. injections during 5 d, produced by Invitrogen Corp. (Carlsbad, California, USA).

LPS Absorption Test

An LPS tolerance test was performed as follows: Fasted mice were gavaged with LPS (300 µg/kg) diluted in water (100 µL) or with water (100 µL). Blood was collected from the cava vein 60 min after gavage. Plasma was separated and frozen [11].

Intracellular Cytokine Analysis and Foxp3 Staining

The cells were obtained from the adipose tissue and analyzed by flow cytometry. For the determination of the frequency of putative regulatory T cells, the adipose tissue mononuclear cells were stained for the surface marker CD4 (Percp) and after for intracellular transcription factor Foxp3 using APC anti-mouse/rat Foxp3 staining (eBioscience, San Diego, California). The cells were acquired in the FACS Calibur Flow cytometer (BD) and analyzed with FlowJo software.

Antibiotics Treatment

Four-week-old WT and TLR2 KO mice were placed on broad spectrum antibiotics (1.0 g/L ampicillin, 1.0 g/L metronidazole, and 0.5 g/L neomycin) in drinking water for 20 d. During this period mice were monitored for food intake and stool microbiota sequencing.

Culture-Based Microbial Analysis of Cecal Contents

Total aerobic and anaerobic bacteria were enumerated in selective media and incubation conditions according to Schumann et al. [74]. In brief, cecal samples were diluted in Ringer medium, and total aerobic and anaerobic bacteria were investigated by plating onto nonselective media: TSS medium (Biomerieux, Lyon, France) for 24 to 48 h at 37°C in aerobic and anaerobic conditions. Bacterial numbers were expressed as colony forming units (CFU)/mg cecal content [75].

Metagenome Profile

Faeces samples were collected in metabolic cages with separated waste collectors, frozen in liquid nitrogen, and kept at −80°C until use. DNA was then extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) and quantified. Libraries were synthesized from 500 ng of total DNA following the Rapid Library Preparation Kit (Roche Applied Science, Mannheim, Germany) instructions. These libraries were analyzed in a Bioanalyzer with a High Sensitive DNA Kit (Agilent Technologies Inc., Santa Clara, California, USA), and equimolar pools were made, titrated, and submitted to large volume PCR, following the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Subsequently, samples were sequenced in GS FLX Titanium, using a GS FLX Titanium PicoTiterPlate Kit combined with a GS FLX Titanium Sequencing Kit XLR70 (Roche Applied Science, Mannheim, Germany). The data obtained from the sequencing were submitted to the MG-RAST server and compared by phylum prevalence among groups [76].

Microbiota Transplantation

Cecal contents were pooled from 3 TLR2 KO mice and age- and gender-matched WT littermates. Cecal extracts were suspended in PBS (2.5 ml per cecum) and were administered (0.1 ml per mouse) immediately to sterilely packed, 4-wk-old, *Bacillus*-associated, WT mice that were obtained from the Central Breeding Center of the State University of Campinas. Transplanted mice were maintained in sterile cages and monitored for body weight [30].

Tissue Extraction, Immunoprecipitation, and Immunoblotting

Mice were anesthetized by intraperitoneal injection of sodium thiopental and used 10–15 min later—i.e., as soon as anesthesia was assured by the loss of pedal and corneal reflexes. In some experiments, 3 or 5 min after insulin injection (3.8 units/kg, intraperitoneally), liver or muscle and white adipose tissue were removed, respectively, and homogenized immediately in extraction buffer at 4°C (1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonyl fluoride, and 0.1 mg of aprotinin/ml) with a Polytron PTA 20 S generator (model PT 10/35; Brinkmann Instruments). Insoluble material was removed by centrifugation for 30 min at 9,000×g in a 70 Ti rotor (Beckman, Fullerton, California) at 4°C. The protein concentrations of the supernatants were determined by the Bradford dye binding method. Aliquots of the resulting supernatants containing 1.0 mg of total protein were used for immunoprecipitation with antibodies against MyD88 overnight at 4°C, followed by SDS-PAGE, transfer to nitrocellulose membranes, and blotting with anti-TLR4. In direct immunoblot experiments, 0.2 mg of protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-UCP1, anti-phospho-JNK, anti-IκBα, anti-phospho-PERK, anti-phospho-AKT, anti-phospho [Ser307]-IRS-1, anti-phospho [Tyr941]-IRS-1 (Tyr), anti-phospho-IR, anti-ZO-1, anti-PGC-1α, anti-phospho [Thr171]-AMPK, and anti-IκB-α. The homogeneity of gel loading was always evaluated by blotting the membranes with antibodies against β-actin, IRS-1, AKT, IR, JNK, PERK, and AMPK as appropriate.

Statistical Analysis

Specific protein bands present on the blots were quantified by densitometry. Mean ± S.E. values obtained from densitometric

scans and from the other experiments were compared utilizing Student's *t* test for paired samples or by repeat-measure analysis of variance (one-way or two-way analysis of variance) followed by post hoc analysis of significance (Bonferroni test) when appropriate. When analyzing non-linear parameters, we used Mann-Whitney test. A $p<0.05$ was accepted as statistically significant.

Supporting Information

Figure S1 Taxonomical characterization of WT gut microbiota. Untreated WT stools were analyzed via 16S rRNA analysis.
(TIF)

Figure S2 TLR2 KO mice exhibit taxonomical alterations in gut microbiota. TLR2 knockout (TLR2−/−) mice stools were analyzed via 16S rRNA analysis.
(TIF)

Figure S3 Bacterial phyla distribution in 4-wk-old-WT (A) or –TLR2−/− mice (B). These analyses were obtained by 16S rRNA sequencing.
(TIF)

Figure S4 Bacterial phyla distribution in 16-wk-old-WT (A) or –TLR2−/− mice (B). These analyses were obtained by 16S rRNA sequencing.
(TIF)

Figure S5 Bacterial phyla distribution in 1-y-old-WT (E) or –TLR2−/− mice (F). These analyses were obtained by 16S rRNA sequencing.
(TIF)

Figure S6 Phosphorylation of the insulin receptor in muscle (A), liver (B), and white adipose tissue (WAT) (C). Tyrosine 172 phosphorylation of AMPK in muscle (D), liver (E), and WAT (F). PGC-1α expression in muscle (G), liver (H), and WAT (I). Insulin receptor and AMPK protein expression in muscle, liver, and WAT (lower panels). Equal protein loading in the gel was confirmed by reblotting the membrane with an anti-β-actin antibody (lower panels). Data are presented from six to eight mice per group, from experiments that were repeated at least three times. All evaluations were made with mice on standard chow. * $p<0.05$ between WT mice with or without insulin stimulus; ** $p<0.05$ between WT and TLR2−/− mice with insulin stimulus.
(TIF)

Figure S7 Cecal samples of TLR2 knockout (TLR2−/−) mice were cultured in aerobic and anaerobic environments with or without the treatment with a mixture of antibiotics (AB) (0.5 g/kg neomycin, 1 g/kg metronidazole, and 1.0 g/kg ampicillin). Aerobic bacteria counts went below the detection limit in the groups treated with the AB. TLR2 knockout (TLR2−/−) mice exhibit taxonomical alterations in gut microbiota after the treatment with a mixture of AB (C), compared with controls, without antibiotics treatment (B). Mice stools were analyzed via 16S rRNA analysis. Data are presented from six to eight mice per group, from experiments that were repeated at least three times. All evaluations were made with mice on standard chow. * $p<0.05$ between aerobic bacteria of TLR2−/− mice with or without AB treatment; ** $p<0.05$ between anaerobic bacteria of TLR2−/− mice with or without AB treatment.
(TIF)

Figure S8 Taxonomical characterization of WT gut microbiota after the treatment with a mixture of antibiotics (AB) (0.5 g/kg neomycin, 1 g/kg metronidazole, and 1.0 g/kg ampicillin). WT+AB stools were analyzed via 16S rRNA analysis.
(TIF)

Figure S9 Taxonomical characterization of TLR2−/− gut microbiota after the treatment with a mixture of antibiotics (AB) (0.5 g/kg neomycin, 1 g/kg metronidazole, and 1.0 g/kg ampicillin). TLR2−/− + AB stools were analyzed via 16S rRNA analysis.
(TIF)

Figure S10 Taxonomical alteration obtained from the treatment with antibiotics individually (0.5 g/kg neomycin, 1 g/kg metronidazole, and 1.0 g/kg ampicillin). TLR2−/− mice treated only with metronidazole (A). TLR2−/− mice treated only with neomycin (B). TLR2−/− mice treated only with ampicillin (C).
(TIF)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: AC MS. Performed the experiments: AC PKP LLA ER MU PP SH AC PV NOSC. Analyzed the data: AC MS JBC. Contributed reagents/materials/analysis tools: RC NOSC JBC MS. Wrote the paper: AC MS.

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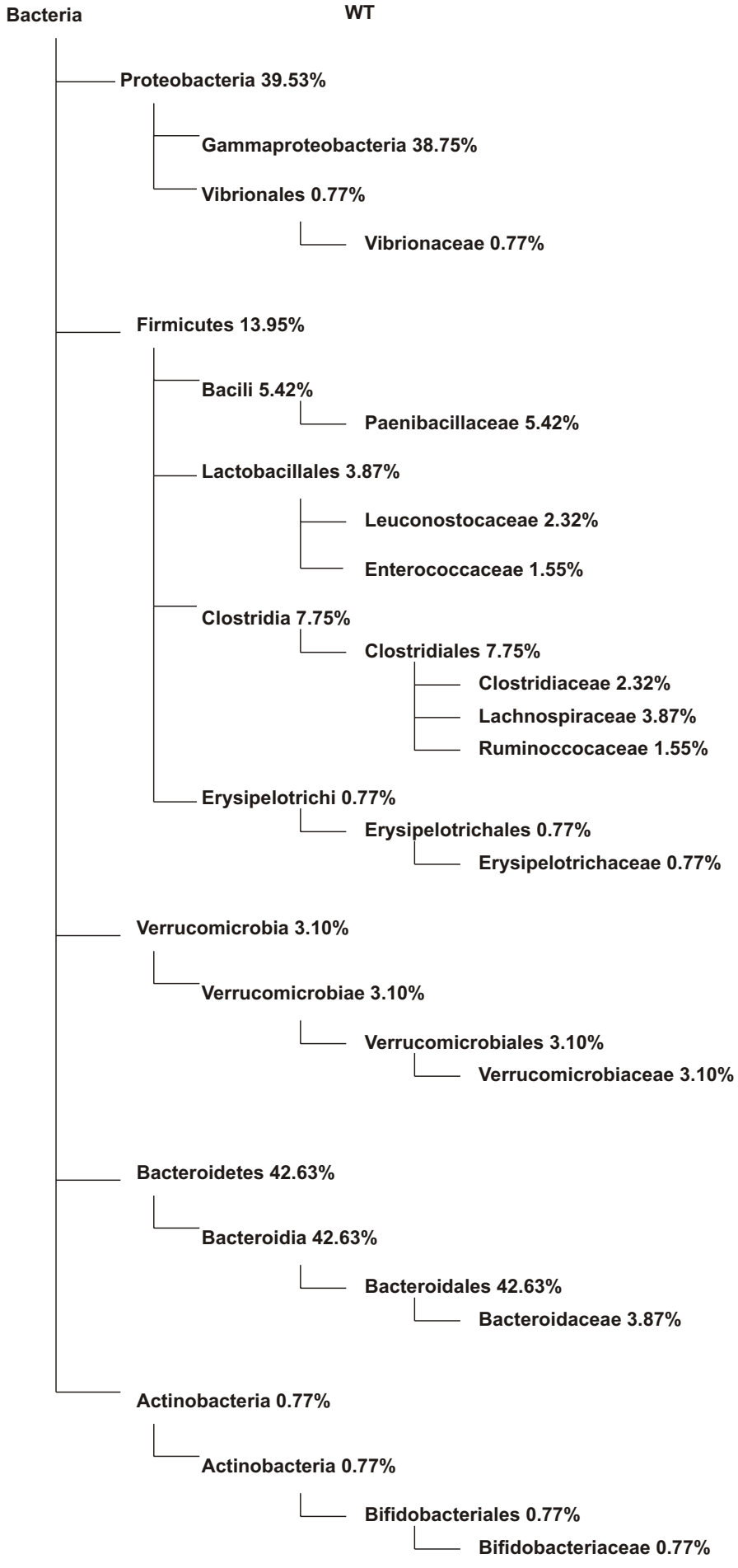
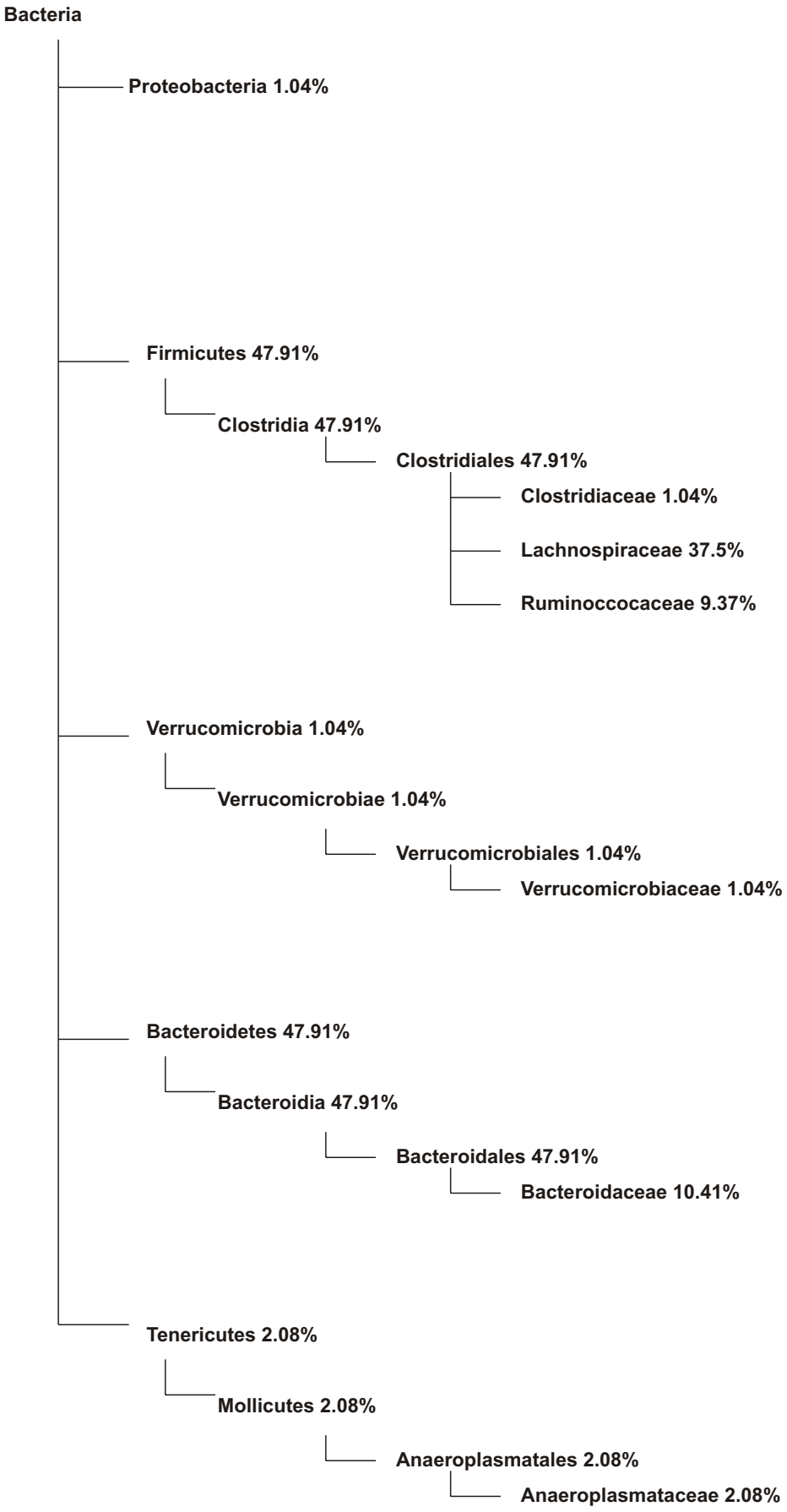
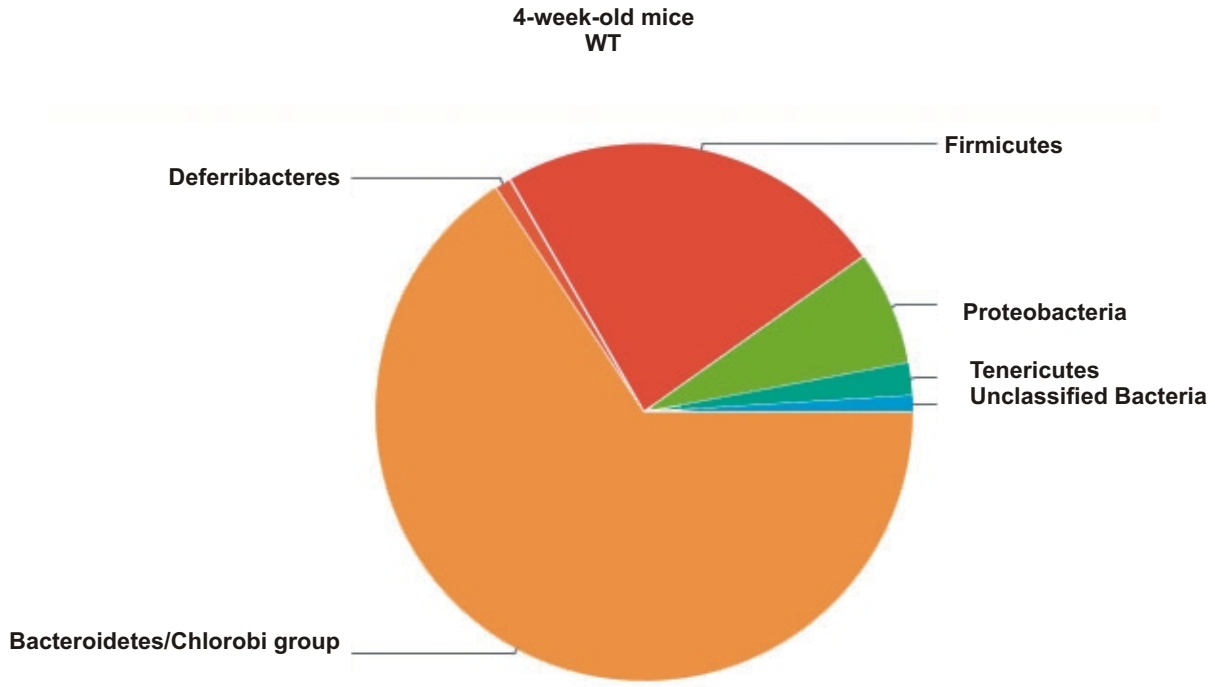
[Material Suplementar]


Bacteria
WT
Proteobacteria 39.53%
Gammaproteobacteria 38.75%
Vibrionales 0.77%
Vibrionaceae 0.77%
Firmicutes 13.95%
Bacili 5.42%
Paenibacillaceae 5.42%
Lactobacillales 3.87%
Leuconostocaceae 2.32%
Enterococcaceae 1.55%
Clostridia 7.75%
Clostridiales 7.75%
Clostridiaceae 2.32%
Lachnospiraceae 3.87%
Ruminoccocaceae 1.55%
Erysipelotrichi 0.77%
Erysipelotrichales 0.77%
Erysipelotrichaceae 0.77%
Verrucomicrobia 3.10%
Verrucomicrobiae 3.10%
Verrucomicrobiales 3.10%
Verrucomicrobiaceae 3.10%
Bacteroidetes 42.63%
Bacteroidia 42.63%
Bacteroidales 42.63%
Bacteroidaceae 3.87%
Actinobacteria 0.77%
Actinobacteria 0.77%
Bifidobacteriales 0.77%
Bifidobacteriaceae 0.77%

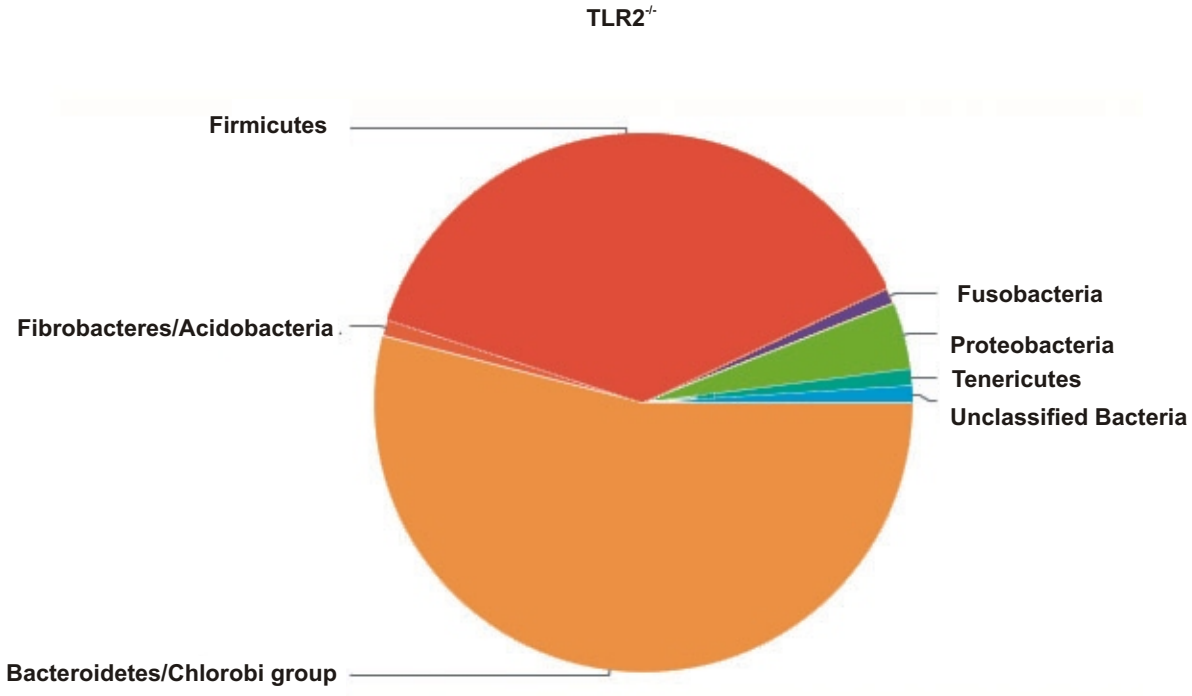
TLR2$^{-/-}$


Bacteria
Proteobacteria 1.04%
Firmicutes 47.91%
Clostridia 47.91%
Clostridiales 47.91%
Clostridiaceae 1.04%
Lachnospiraceae 37.5%
Ruminoccocaceae 9.37%
Verrucomicrobia 1.04%
Verrucomicrobiae 1.04%
Verrucomicrobiales 1.04%
Verrucomicrobiaceae 1.04%
Bacteroidetes 47.91%
Bacteroidia 47.91%
Bacteroidales 47.91%
Bacteroidaceae 10.41%
Tenericutes 2.08%
Mollicutes 2.08%
Anaeroplasmatales 2.08%
Anaeroplasmataceae 2.08%

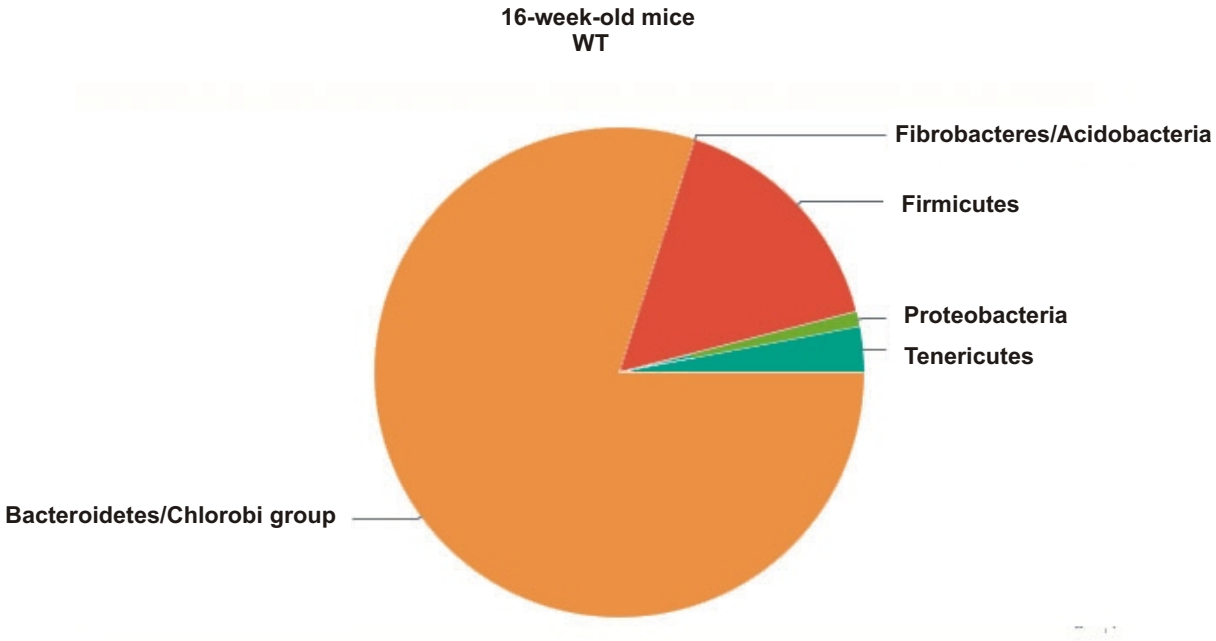
A


4-week-old mice
WT
Firmicutes
Deferribacteres
Proteobacteria
Tenericutes
Unclassified Bacteria
Bacteroidetes/Chlorobi group

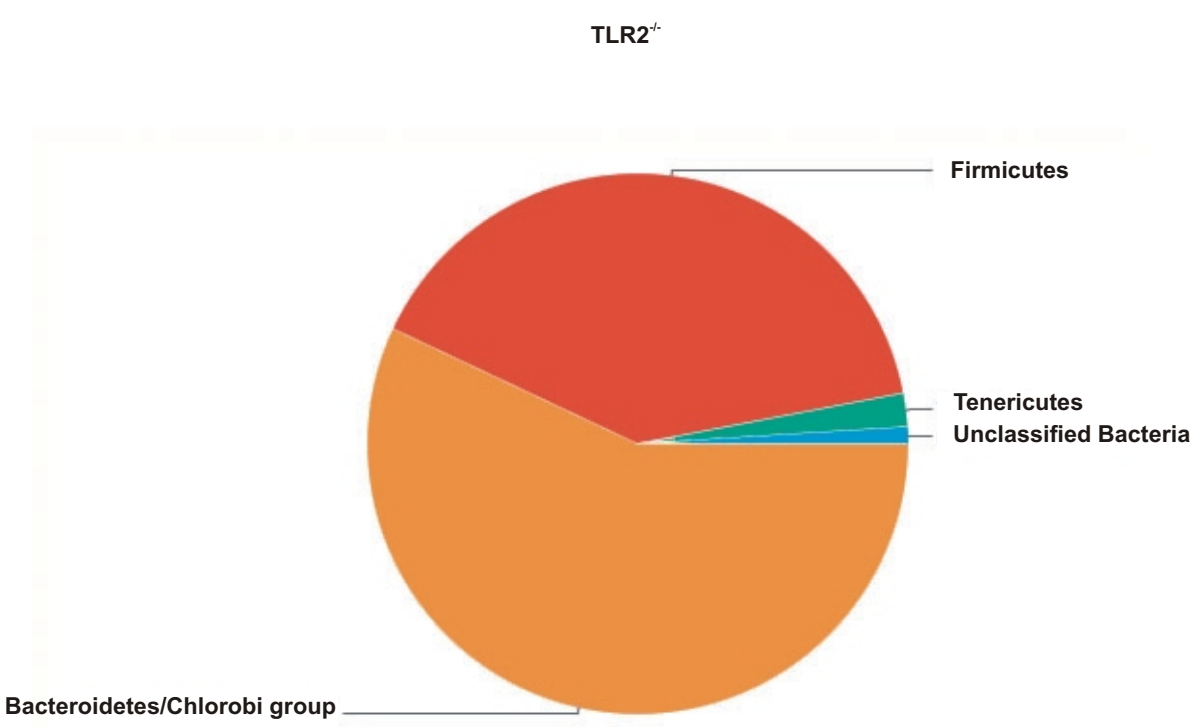
B


TLR2-/-
Firmicutes
Fusobacteria
Fibrobacteres/Acidobacteria
Proteobacteria
Tenericutes
Unclassified Bacteria
Bacteroidetes/Chlorobi group

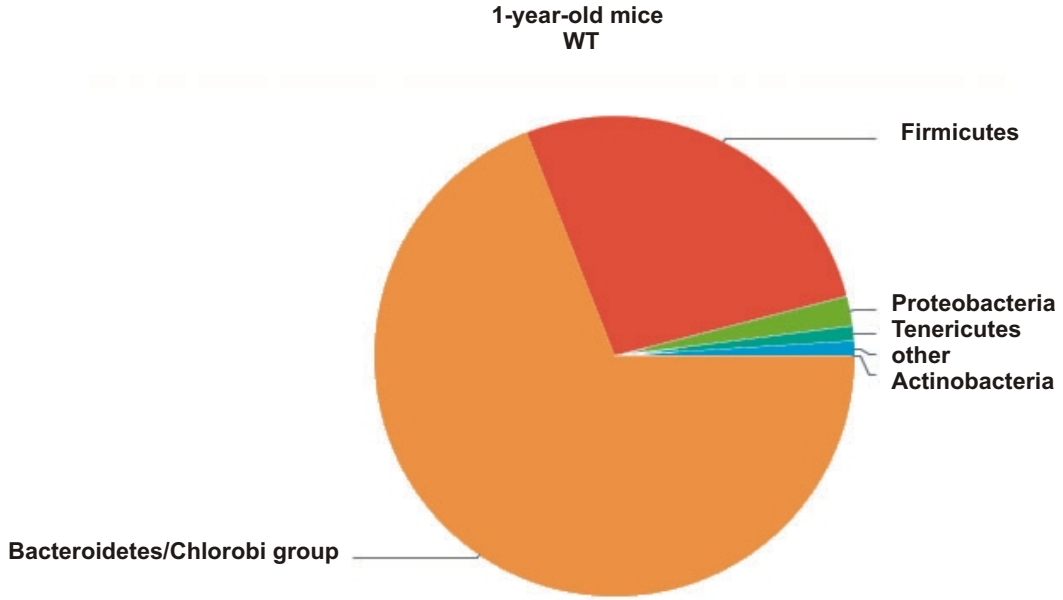
A


16-week-old mice
WT
Fibrobacteres/Acidobacteria
Firmicutes
Proteobacteria
Tenericutes
Bacteroidetes/Chlorobi group

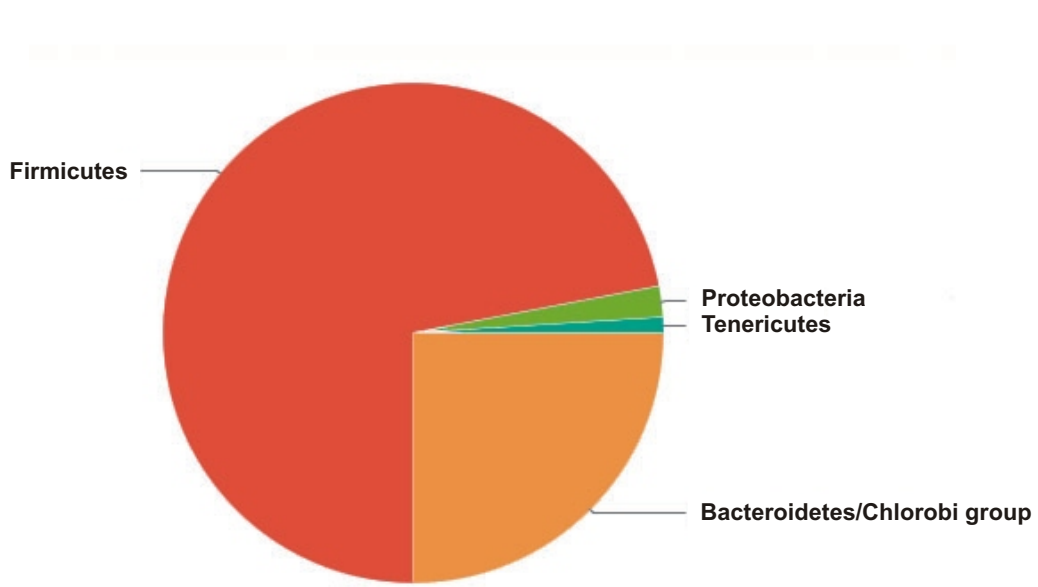
B

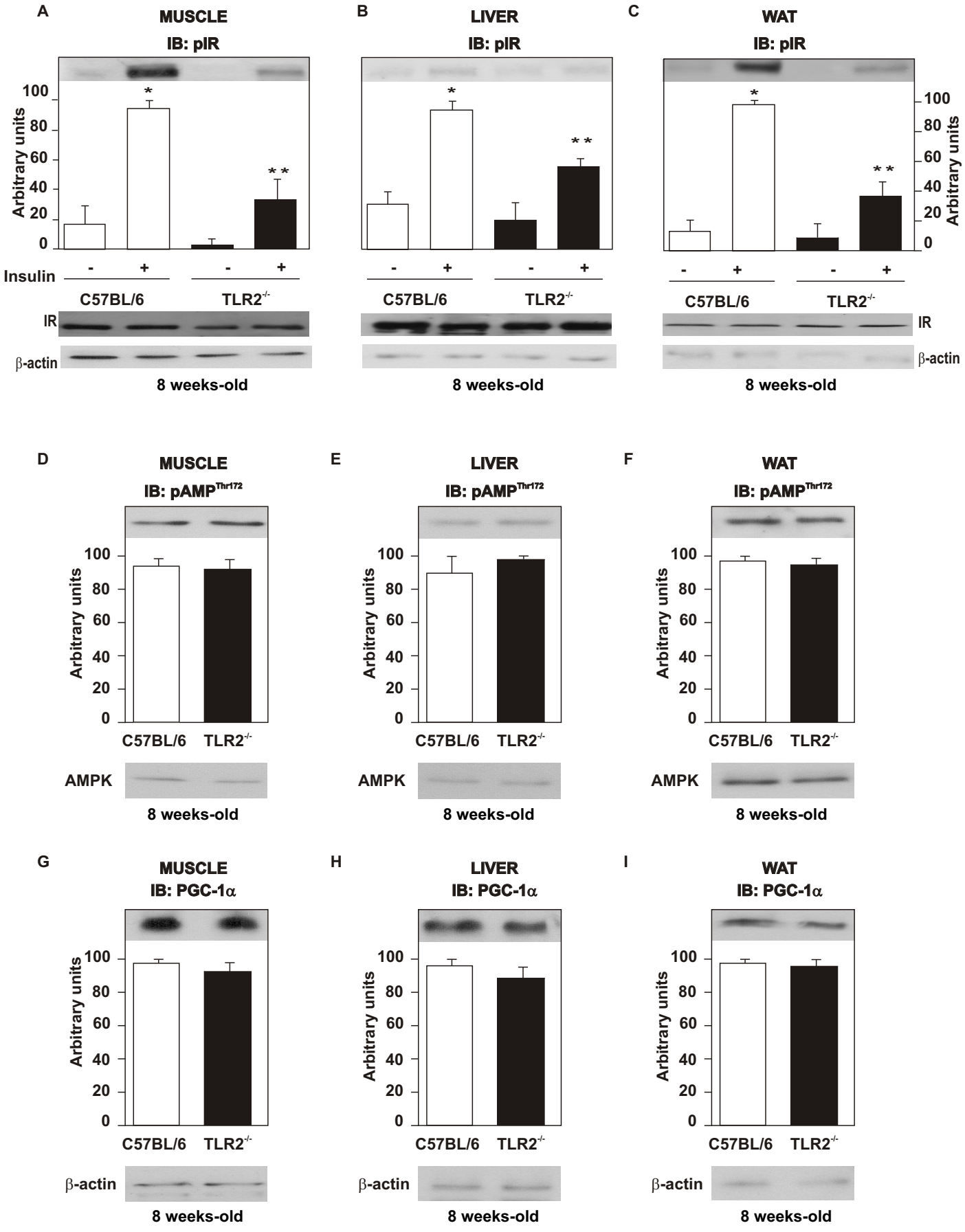

TLR2-/-
Firmicutes
Tenericutes
Unclassified Bacteria
Bacteroidetes/Chlorobi group

A

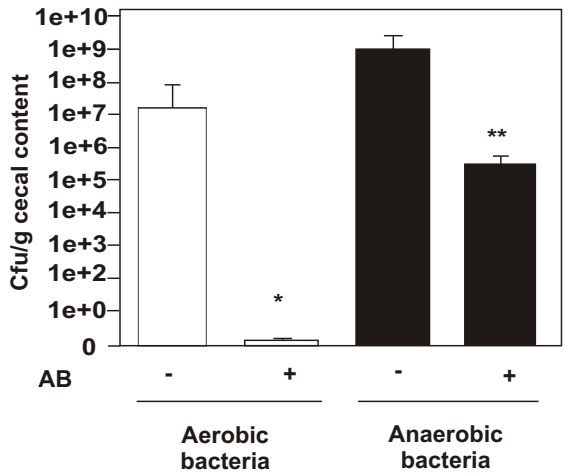

1-year-old mice
WT
Firmicutes
Proteobacteria
Tenericutes
other
Actinobacteria
Bacteroidetes/Chlorobi group

B

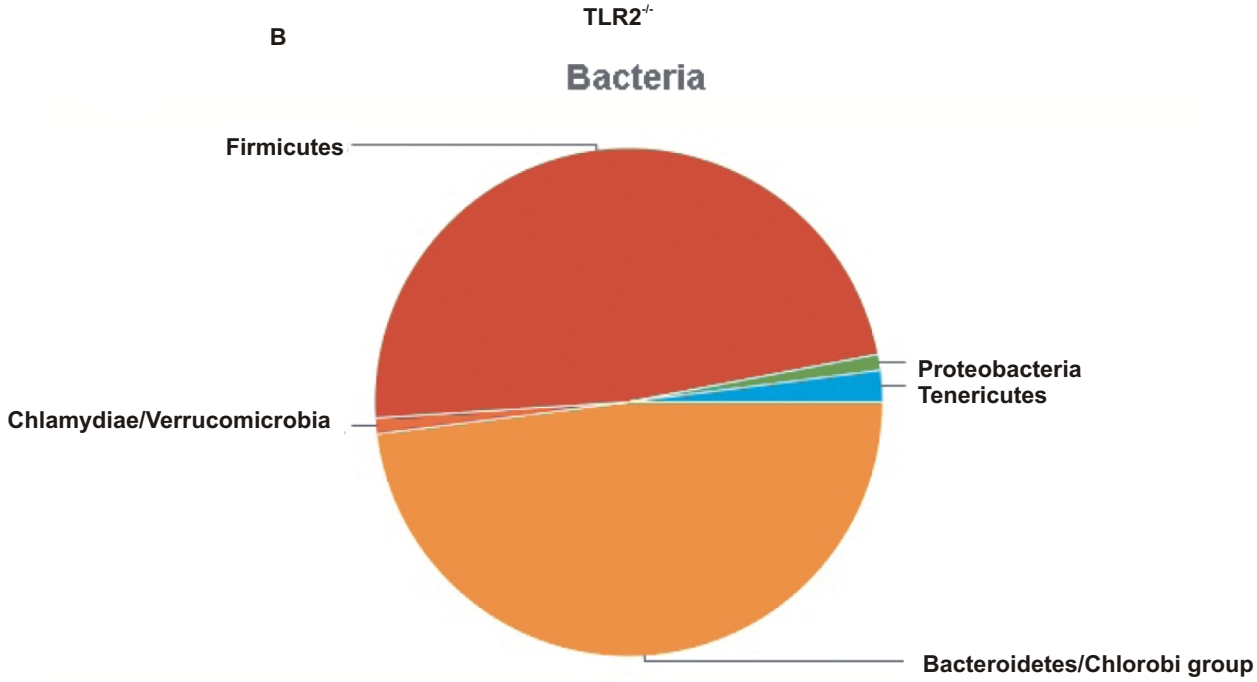

TLR2-/-
Firmicutes
Proteobacteria
Tenericutes
Bacteroidetes/Chlorobi group


A
MUSCLE
IB: pIR
B
LIVER
IB: pIR
C
WAT
IB: pIR
Arbitrary units
100
80
60
40
20
0
*
**
Insulin
-
+
C57BL/6
TLR2-/-
IR
β-actin
8 weeks-old
D
MUSCLE
IB: pAMPThr172
E
LIVER
IB: pAMPThr172
F
WAT
IB: pAMPThr172
AMPK
G
MUSCLE
IB: PGC-1α
H
LIVER
IB: PGC-1α
I
WAT
IB: PGC-1α

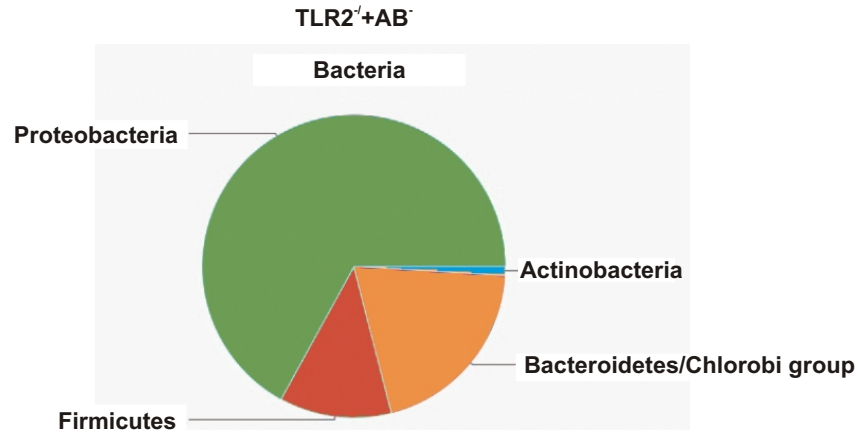
A

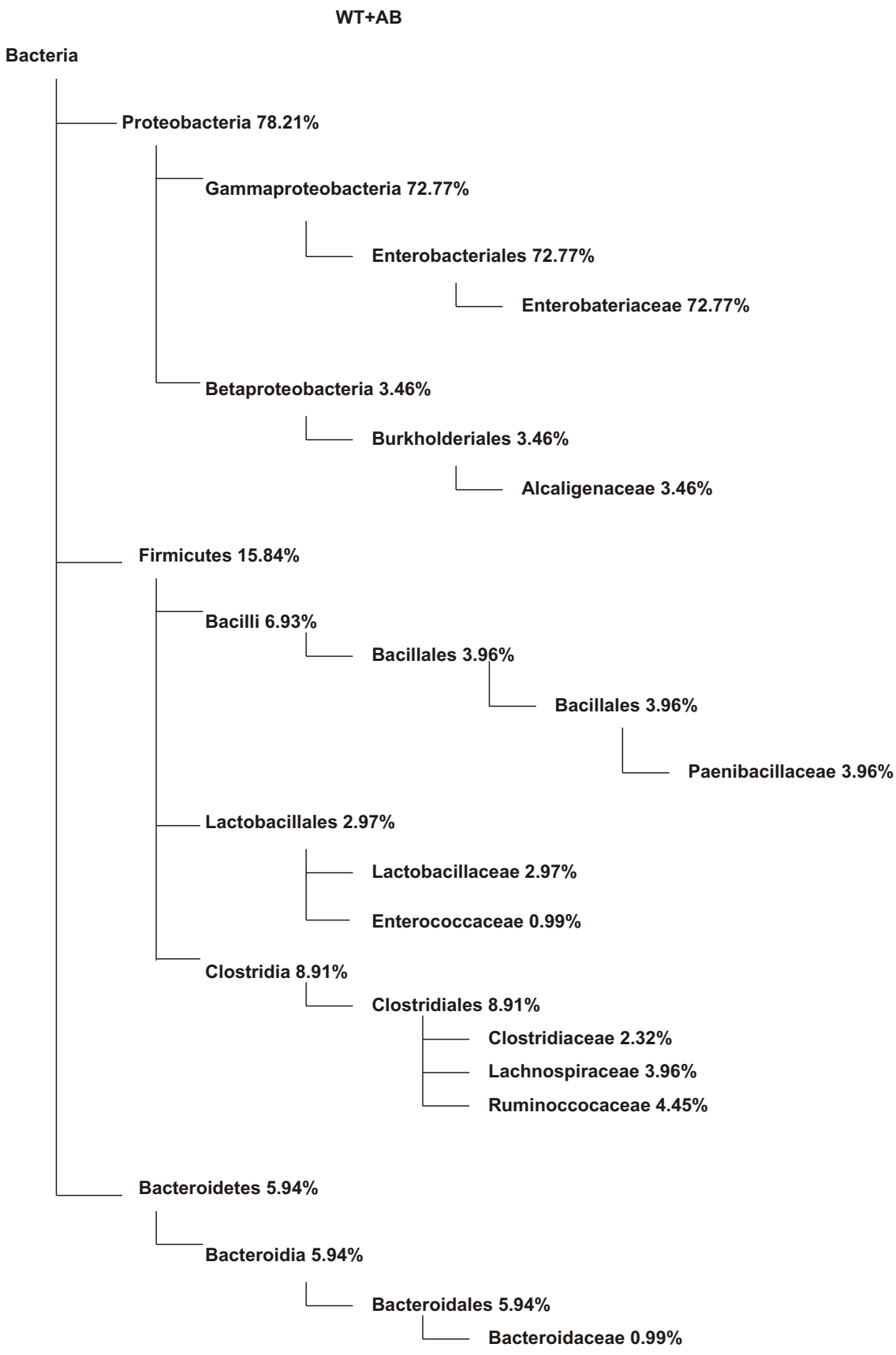

1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+0
0
Cfu/g cecal content
*
**
AB
-
+
-
+
Aerobic bacteria
Anaerobic bacteria

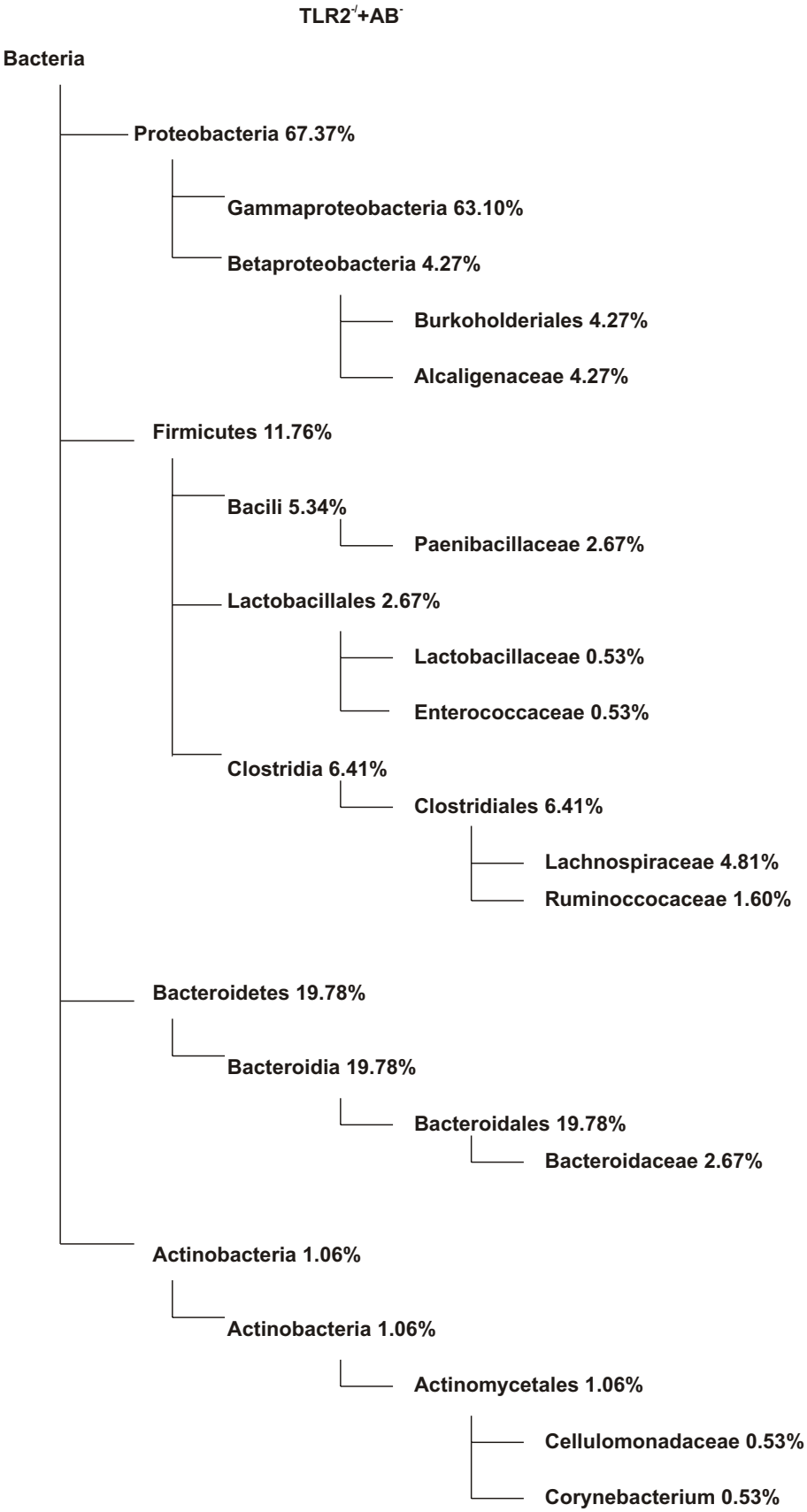
B

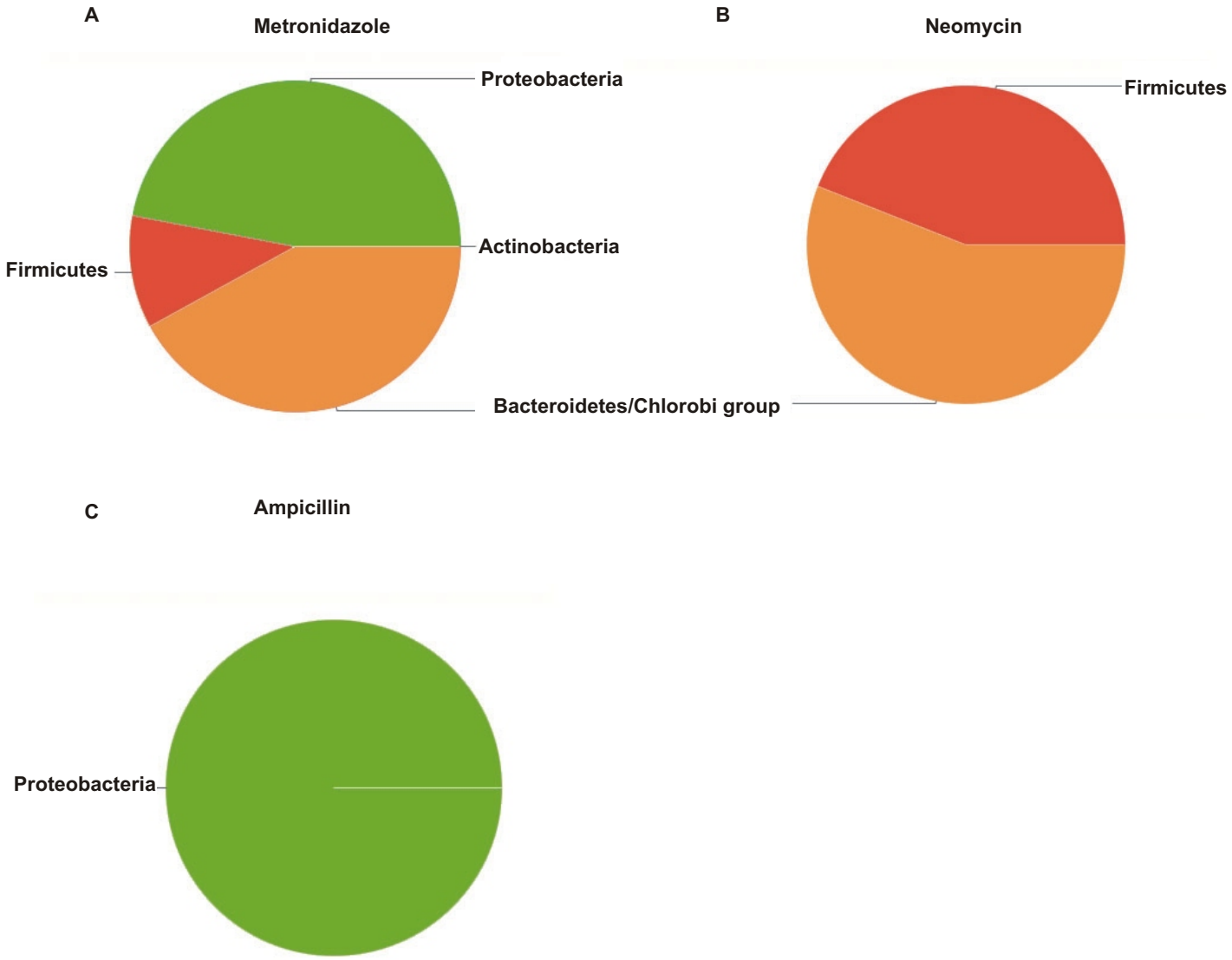

TLR2-/-
Bacteria
Firmicutes
Proteobacteria
Tenericutes
Chlamydiae/Verrucomicrobia
Bacteroidetes/Chlorobi group

C


TLR2-/-+AB-
Bacteria
Proteobacteria
Actinobacteria
Bacteroidetes/Chlorobi group
Firmicutes


WT+AB
Bacteria
Proteobacteria 78.21%
Gammaproteobacteria 72.77%
Enterobacteriales 72.77%
Enterobateriaceae 72.77%
Betaproteobacteria 3.46%
Burkholderiales 3.46%
Alcaligenaceae 3.46%
Firmicutes 15.84%
Bacilli 6.93%
Bacillales 3.96%
Bacillales 3.96%
Paenibacillaceae 3.96%
Lactobacillales 2.97%
Lactobacillaceae 2.97%
Enterococcaceae 0.99%
Clostridia 8.91%
Clostridiales 8.91%
Clostridiaceae 2.32%
Lachnospiraceae 3.96%
Ruminoccocaceae 4.45%
Bacteroidetes 5.94%
Bacteroidia 5.94%
Bacteroidales 5.94%
Bacteroidaceae 0.99%


TLR2-/+AB-
Bacteria
Proteobacteria 67.37%
Gammaproteobacteria 63.10%
Betaproteobacteria 4.27%
Burkoholderiales 4.27%
Alcaligenaceae 4.27%
Firmicutes 11.76%
Bacili 5.34%
Paenibacillaceae 2.67%
Lactobacillales 2.67%
Lactobacillaceae 0.53%
Enterococcaceae 0.53%
Clostridia 6.41%
Clostridiales 6.41%
Lachnospiraceae 4.81%
Ruminoccocaceae 1.60%
Bacteroidetes 19.78%
Bacteroidia 19.78%
Bacteroidales 19.78%
Bacteroidaceae 2.67%
Actinobacteria 1.06%
Actinobacteria 1.06%
Actinomycetales 1.06%
Cellulomonadaceae 0.53%
Corynebacterium 0.53%


A
Metronidazole
Proteobacteria
Actinobacteria
Firmicutes
B
Neomycin
Firmicutes
Bacteroidetes/Chlorobi group
C
Ampicillin
Proteobacteria

[Discussão geral]

Discussão geral

Atualmente, considera-se que os fatores ambientais e a genética do hospedeiro interagem para controlar a aquisição e a estabilidade da microbiota intestinal. Por outro lado, ambiente, genética do hospedeiro e microbiota interagem para manter a homeostase do trato gastrintestinal, controle de peso e sensibilidade à insulina (17). Logicamente, a modificação de um ou mais desses três componentes pode levar ao desenvolvimento da resistência à insulina e obesidade. Os resultados do presente estudo demonstraram que os camundongos KO para TLR2 em biotérios convencionais de nosso centro de criação possuem resistência à insulina e intolerância à glicose associadas com alterações na composição da microbiota intestinal, que mostrou aumento da abundância relativa de Firmicutes e Bacteroidetes, bem como uma redução da abundância relativa de Proteobacteria, em comparação com os controles. A resistência à insulina dos KO para TLR2 foi acompanhada de uma redução da modulação da sinalização da insulina no fígado, músculo e tecido adiposo, associada com aumento do estresse de retículo endoplasmático. Essas alterações metabólicas foram caracterizadas em camundongos KO para TLR2 com oito semanas de idade, quando eles possuíam pesos corpóreos semelhantes aos dos controles. Como demonstrado em outros modelos animais (90, 91), a resistência à insulina precede o desenvolvimento da obesidade, sendo que o aumento do peso corpóreo dos KO, em comparação com os controles, é observado após a vigésima semana de idade.

No entanto, estudos anteriores (84, 92) mostraram que camundongos KO para TLR2 apresentam redução do peso corpóreo e da adiposidade, estão protegidos da resistência à insulina, ganham menos peso do que os controles

quando alimentados com dieta hiperlipídica e estão protegidos de outras comorbidades relacionadas (93, 94). Acreditamos que a diferença principal entre esses estudos e o nosso esteja relacionada com a microbiota intestinal. Deve-se levar em consideração que, embora esses animais tenham a mesma deficiência genética, eles foram criados em diferentes locais e alimentados com dieta de diferentes fontes, o que certamente tem um papel no estabelecimento e manutenção da microbiota intestinal.

Da mesma maneira, quando o TLR2 tem sua expressão inibida de forma aguda, observa-se melhora na sensibilidade e sinalização da insulina (41, 86). O estudo de J.J. Senn (41) mostrou que a inibição desse receptor com uso de anticorpo monoclonal em miotubos tratados com palmitato bloqueou a produção de IL-6 e restaurou a fosforilação da AKT, reduzindo a resistência à insulina induzida por ácido graxo saturado através da ativação do TLR2. Nosso estudo, que realizou inibição aguda do TLR2 *in vivo*, por meio de utilização de oligonucleotídeo antisense para TLR2, também foi capaz de reverter a resistência à insulina induzida por dieta hiperlipídica em músculo e tecido adiposo de camundongos, reduzindo a ativação de vias como IKK e JNK, e reduzindo a fosforilação inibitória em serina 307 do IRS-1. Esse tratamento aumentou também a sensibilidade à insulina, como averiguado pelo teste de tolerância à insulina (86). Essa inibição, por ser aguda, de apenas quatro dias, provavelmente não foi capaz de gerar adaptação do organismo, como, por exemplo, por meio de ativações compensatórias, nem alterações na microbiota intestinal, de modo que o fenótipo gerado é de melhora da sensibilidade e sinalização da insulina.

Os mecanismos pelos quais os camundongos KO para TLR2 apresentaram resistência à insulina e posterior obesidade também foram investigados. A microbiota intestinal do animal KO para TLR2 possuía alguma semelhanças com a encontrada em animais e humanos obesos, com aumento na proporção de Firmicutes (60, 95). Esse tipo de microbiota é normalmente associado com um aumento da capacidade de extração de energia da dieta (58). Isso pode contribuir para explicar a obesidade observada, mas não explica por que esses animais são mais resistentes à insulina muitas semanas antes de eles começarem a ganhar mais peso do que os controles. Além disso, demonstrou-se que camundongos livres de microorganismos (que ganham menos peso corpóreo em dieta hiperlipídica) e os mantidos em biotérios convencionais possuem conteúdos energéticos semelhantes em suas fezes, sugerindo que outros mecanismos podem ter importante papel na resistência à insulina e na obesidade induzidas por alterações na microbiota intestinal (96).

Apesar de na maioria dos estudos anteriores a microbiota intestinal não ter sido investigada, podemos sugerir que a deficiência de TLR2 associada com ambientes diferentes pode induzir fenótipos distintos, provavelmente induzidos por microbiotas diversas. Kellermayer *at al.* mostraram que a proporção de Firmicutes encontrada em camundongos KO para TLR2 foi menor do que no tipo selvagem (WT, do inglês *wild type*), enquanto a proporção de Bacteroidetes foi maior (97). Em nosso estudo, mostramos que os camundongos KO para TLR2 apresentaram o oposto, com maior proporção de Firmicutes e menor proporção de Bacteroidetes, em comparação com os WT. Dessa maneira, é possível que, em outros estudos publicados, as proporções de filos sejam diferentes, comparando com as proporções

encontradas em nosso estudo, o que pode influenciar diferentemente no fenótipo observado. Esses resultados reforçam a importância do ambiente e do sistema imune inato como reguladores chaves da microbiota intestinal e sugerem que a condição genética, que pode, por si mesma, prevenir a resistência à insulina em algumas condições, pode também subjugar o efeito protetor contra a resistência à insulina em outras condições ambientais, induzindo mais ganho de peso, provavelmente devido a diferenças no comportamento metabólico de um mesmo animal, quando analisado em diferentes ambientes, o que contribui para explicar diferenças no comportamento metabólico entre animais com o mesmo genótipo ou com a mesma alteração genética.

Alguns estudos sugerem ainda que a microbiota intestinal pode contribuir para a obesidade ao induzir a redução da oxidação de gordura e o aumento do estoque lipídico (96), associados com uma relativa redução da expressão de PGC1-α e da fosforilação da AMPK. Esse mecanismo é menos provável em nosso modelo animal, dado que o quociente respiratório dos camundongos KO para TLR2 foi idêntico ao dos controles, mostrando que eles oxidam lipídios na mesma proporção dos controles, e que os níveis teciduais de PGC1-α e de fosforilação de AMPK mostraram-se semelhantes em fígado e músculo de controles e de KO para TLR2.

Outro mecanismo possível que pode induzir a resistência à insulina na obesidade é o aumento dos níveis de LPS, o que é observado em camundongos alimentados com dieta hiperlipídica (98, 99). Notavelmente, embora os camundongos KO para TLR2 tenham sido alimentados com ração padrão, eles apresentaram níveis circulantes de LPS aumentados. Como a

microbiota desses camundongos tinha uma maior predominância de Firmicutes, que são bactérias gram-positivas, e não possuem LPS em sua membrana externa, o aumento dos níveis circulantes de LPS não seria conseqüência de uma microbiota que produz mais LPS. Não obstante, a microbiota observada em obesos e também em camundongos KO para TLR2 pode aumentar a permeabilidade intestinal e a absorção de LPS (100-102). De forma importante, como observado em camundongos obesos, que apresentam uma significativa redução da proporção de Bifidobacteria (103, 104), na microbiota de camundongos KO para TLR2 magros, esse gênero estava reduzido comparado com os controles. Nesse sentido, a suplementação com Bifidobacteria tem sido associada à melhora da função da barreira intestinal e aos níveis reduzidos de LPS (105-107). A fim de provar que o aumento dos níveis circulantes de LPS nos camundongos KO para TLR2 estava relacionado ao aumento da permeabilidade intestinal, administramos LPS oralmente a esses camundongos e observamos que, além dos níveis basais aumentados de LPS, esses animais também apresentaram aumento do pico de LPS uma hora após a gavagem com LPS.

Resultados anteriores mostraram que o TLR2 regula a integridade de junções intercelulares da barreira epitelial do intestino e que a deficiência desse receptor predispõe alterações na função de barreira, levando à perpetuação da inflamação mucosa (108, 109). Nesse sentido, nossos estudos também demonstraram que, em camundongos KO para TLR2, há uma redução da expressão de ZO-1 em intestino delgado e cólon, reforçando que existem alterações na integridade epitelial e na permeabilidade intestinal desses camundongos. Juntos, esses resultados sugerem que as interações entre a

predisposição dos KO para TLR2 a terem alterações na barreira intestinal com a microbiota intestinal podem ter importante papel no aumento dos níveis circulantes de LPS.

De acordo com as alterações na permeabilidade intestinal, Kellermayer *et. al.* investigaram recentemente as conseqüências epigenômicas e metagenômicas da deficiência do *Tlr2* na mucosa do cólon de camundongos, a fim de compreenderem as vias biológicas que modelam a interface entre microbiota intestinal e hospedeiro mamífero. Os resultados mostraram modificações epigenéticas e transcriptômicas associadas com alterações na composição microbiana da mucosa e que a abundância de muitas espécies bacterianas foram diferentes entre WT e KO para TLR2. A expressão de genes envolvidos no sistema imune foi alterada na mucosa do cólon de camundongos KO para TLR2, o que se correlaciona com mudanças de metilação do DNA. Esse estudo pioneiro demonstra que alterações significativas da microbiota associam-se com mudanças epigenéticas influenciadas pelo genoma do hospedeiro (110).

A fim de se confirmar que a microbiota intestinal estava induzindo a resistência à insulina em camundongos KO para TLR2, tratamos esses animais com antibióticos por 15 dias e mostramos que esse tratamento reduziu dramaticamente a microbiota intestinal, alterando também sua composição. Em paralelo, houve uma melhora na ação da insulina, caracterizada por um aumento na taxa de infusão de glicose durante o clamp glicêmico, bem como uma melhora na sinalização da insulina em fígado, músculo e tecido adiposo. Em camundongos KO para TLR2 tratados com antibióticos, observamos também uma grande redução dos níveis de LPS. Ao realizarmos o transplante

de microbiota de KO para TLR2 para camundongos WT associados com *Bacillus,* que são colonizados apenas pelo gênero *Bacillus* e são capazes de receberem microbiota distinta de outro camundongo, a composição complexa do organismo doador do transplante foi preservada. A microbiota proveniente de camundongos KO para TLR2 transplantada conferiu maior ganho de peso, intolerância à glicose, sinalização e sensibilidade à insulina reduzidas, com aumento dos níveis circulantes de LPS. Esses resultados reforçam a hipótese de que a microbiota de camundongos KO para TLR2 é capaz de induzir alterações na permeabilidade intestinal, aumentando os níveis séricos de LPS, associados à resistência à insulina.

O aumento dos níveis de LPS pode induzir a resistência à insulina por contrarregulação da sinalização da insulina, como demonstrado anteriormente (26, 31, 98). Entretanto, a resistência à insulina observada em camundongos KO para TLR2 tem características singulares. Observou-se aumento da ativação do TLR4 em fígado, músculo e tecido adiposo, associado com estresse de retículo endoplasmático e ativação da JNK, sem, porém, ativação da via IKKβ-IκB-NFκB. Foi previamente descrito que existe uma cooperação na sinalização de TLR4 e TLR2. Essa cooperação é evidenciada quando se injeta LPS em camundongos KO para TLR2. Após o primeiro bolus de LPS, os camundongos KO para TLR2 mostram um sinal robusto para genes que codificam para proteínas do sistema imune inato no cérebro. Porém, uma segunda infusão de LPS falha no desencadeamento da expressão de TNF-α em camundongos KO para TLR2. Esses resultados indicam que o TLR2 está envolvido na segunda onda de expressão de TNF-α após estímulo com LPS, e que há uma elegante cooperação entre TLR2 e TLR4 (88). Nossos resultados

estendem esses achados ao mostrarem que uma elevação crônica dos níveis de LPS em camundongos KO para TLR2 não foi capaz de aumentar a ativação da via IKK/IkB/NF-kB, bem como a produção de TNF-α e de IL-6, mas induziu aumento da ativação da JNK em fígado, músculo e tecido adiposo desses camundongos. Esses resultados sugerem que uma ativação crônica de TLR4 por baixas doses de LPS é suficiente para aumentar a ativação da JNK, mas a ativação da via IKK/IkB/NF-kB pode também depender de uma cooperação entre TLR2 e TLR4.

A ausência da ativação da via NFκB e os níveis reduzidos de TNFα e de IL-6 fazem da resistência à insulina dos camundongos KO para TLR2 diferente daquela observada em camundongos com obesidade induzida por dieta ou em animais ob/ob. Sugerimos, portanto, que o aumento de níveis circulantes de LPS foi responsável pela ativação do TLR4, pela indução do estresse de retículo endoplasmático e pela ativação da JNK, acompanhados pelo aumento da fosforilação em serina 307 do IRS-1 no fígado, músculo e tecido adiposo, levando a uma redução da sensibilidade e sinalização da insulina, conferindo o fenótipo observado nos camundongos KO para TLR2. Fosforilação do IRS-1 em resíduos de serina interfere na subseqüente fosforilação em tirosina estimulada por insulina do IRS-1 pelo IR (111), e o IRS-1 pode também mediar a inibição da atividade tirosina quinase do receptor de insulina (26), bem como a sinalização subseqüente, como a fosforilação da AKT. A via de sinalização da insulina é crucial para os efeitos da insulina no metabolismo da glicose (112). O bloqueio farmacológico ou genético do TLR4, do estresse de retículo endoplasmático ou da JNK melhora a ação e a sinalização da insulina em

camundongos KO para TLR2, confirmando que essa seqüência de eventos tem um importante papel para a resistência à insulina desses animais.

Células T regulatórias, um pequeno conjunto de linfócitos T, são reconhecidas como uma das mais importantes defesas do corpo contra respostas imunes inapropriadas (113, 114) e podem influenciar as atividades de células do sistema imune inato (115-117). Estudos anteriores mostraram que células T regulatórias encontram-se bastante aumentadas em tecido adiposo abdominal de camundongos controles, e reduzidas nesse tecido de modelos de obesidade. Essa redução de células T regulatórias quando da obesidade influencia o estado inflamatório do tecido adiposo e certamente contribui para a resistência à insulina. Nossos achados, mostrando que, nos camundongos KO para TLR2, há uma redução das células T regulatórias no tecido adiposo visceral, sugerem que essa modulação pode contribuir para a resistência à insulina observada nesses animais.

Tem sido estabelecido que o desenvolvimento da obesidade e da resistência à insulina em humanos é promovido pela dieta hiperlipídica (DH). Ao alimentarmos camundongos KO para TLR2 com dieta hiperlipídica por 8 semanas, houve um pronunciado ganho de peso e aumento da glicemia de jejum, com níveis acima de 400mg/dL duas horas após iniciado o teste de tolerância à glicose, demonstrando que esses animais desenvolvem não apenas uma forma mais severa de resistência à insulina, mas também diabetes. As alterações na sinalização da insulina em tecidos também mostraram um prejuízo, em paralelo com um aumento da ativação da JNK em comparação com controles alimentados com DH. De forma interessante, a ausência da ativação da via IKKβ-IκB-NFκB, descrita para camundongos KO

para TLR2 alimentados com ração padrão, foi observada nesses camundongos em DH. Esses resultados demonstram que a resistência à insulina e posterior aumento do peso corpóreo observado em KO para TLR2 é exacerbado com a DH.

Um estudo recente demonstrou que camundongos deficientes em TLR5 exibem hiperfagia, hiperlipidemia, resistência à insulina e aumento da adiposidade (89). Essas alterações metabólicas correlacionam-se com mudanças na composição da microbiota intestinal. Nosso modelo, embora mostre características semelhantes, apresentou aspectos que sugerem mecanismos operantes diferentes aos dos camundongos deficientes para TLR5 ou TLR2. Primeiramente, os camundongos KO para TLR2 deste estudo não apresentaram hiperfagia, e as diferenças de peso corpóreo iniciam-se apenas quando esses animais têm dezesseis semanas de idade. Em camundongos KO para TLR5, a resistência à insulina não depende de TLR4, enquanto que, em KO para TLR2, há um aumento dos níveis circulantes de LPS e de ativação do TLR4. É possível que essas diferenças não apenas representem alterações genéticas, mas também diferenças nas microbiota intestinal entre esses camundongos.

Em suma, nós podemos sugerir que a perda do TLR2 em camundongos de biotérios convencionais resulta em um fenótipo semelhante ao observado na síndrome metabólica, caracterizado por uma alteração da microbiota intestinal, que induz a resistência à insulina, a inflamação subclínica associada ao estresse de retículo endoplasmático, a intolerância à glicose e posterior obesidade, o que é reproduzido em camundongos WT por transplante de microbiota e pode ser revertido com a utilização de antibióticos. Nossos

resultados enfatizam o papel da microbiota na complexa rede de interações moleculares e celulares que ligam genótipo e fenótipo e têm potenciais implicações para um grande número de doenças humanas envolvendo obesidade, diabetes ou alterações imunológicas.

[Conclusão geral]

Conclusão geral

- É possível que a microbiota dos camundongos deficientes para TLR2 sejam um dos mecanismos responsáveis por induzir o estado de resistência à insulina;

- A ativação do estresse de retículo endoplasmático e do TLR4 são mecanismos importantes para a indução da resistência à insulina em camundongos deficientes para TLR2;

- A microbiota intestinal mostra-se como tendo um papel de conexão entre genótipo e fenótipo e no desencadeamento de desordens como obesidade e diabetes

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Dear Andrea Moro Caricilli

RE: Inhibition of toll-like receptor 2 expression improves insulin sensitivity and signaling in muscle and white adipose tissue of mice fed a high-fat diet. *J Endocrinol*. 2008 Dec;**199**(3):399-406. Caricilli AM, Nascimento PH, Pauli JR, Tsukumo DM, Velloso LA, Carvalheira JB, Saad MJ.

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ANEXO III

Artigo publicado:

"Modulation of Hypothalamic PTP1B in TNF-alpha-induced insulin and leptin resistance", de autoria de **Picardi** PK, Caricilli AM, de Abreu LL, Carvalheira JB, Velloso LA, Saad MJ., FEBS Lett. 2010 Jul 16;584(14):3179-84. Epub 2010 Jun 2.

Artigos submetidos:

"Topical Insulin Accelerates Wound Healing in Diabetes by Enhancing AKT and ERK Pathway: A Double-Blind Placebo-Controlled Clinical Trial" à revista PLoS ONE, revisado e ressubmetido. Autores: Lima MH, Caricilli AM, de Abreu LL, Araújo EP, Pelegrinelli F, Thirone ACP, Tsukumo DM, Moraes MA, Carvalheira JB, Velloso LA, Saad MJA.

"Tub has a Key Role in Insulin and Leptin Signaling/Action in vivo in Hypothalamic Nuclei, submetido ao Diabetes". Autores: Patricia O. Prada, Paula G.F. Quaresma, Andrea M. Caricilli, Andressa C. Santos, Dioze Guadagnini, Joseane Morari, Laís Weissmann, Eduardo R. Ropelle, Jose Barreto C. Carvalheira, Licio A. Velloso and Mario J. Saad

"Effect of Treatment with Fenofibrate and Insulin on Cutaneous Wound Healing in Streptozotocin-Diabetic Rats", submetido ao Wound Repair and Regeneration. Autores: de Abreu LL, Caricilli AM, Picardi PK, Razolli D, Amaral MEC, Esquisatto MAM, Araújo EP, Velloso LA, Saad MJA, Lima MHM

"Targeted Disruption of Inducible Nitric Oxide Synthase Protects Against Aging-Linked S-Nitrosylation and Insulin Resistance in Muscle", submetido ao Aging Cell. Autores: Ropelle E, Pauli JR, Cintra D, da Silva A, de Souza CT, Guadagnini D, Carvalho B, Hirabara S, Caricilli AM, Carvalho-Filho MA, Velloso LA, Saad MJA, Carvalheira JB.

"Inhibition of hypothalamic inflammation reverts diet-induced insulin resistance in the liver", submetido ao Diabetes. Autores: Marciane Milanski, Ana P. Arruda, Andressa Coope, Letícia M. Ignacio-Souza, Erika A. Roman, Carla Nuñes, Talita Romanatto, Livia B. Pascoal, Andrea M. Caricilli, Marcio A. Torsoni, Patricia O. Prada, Mario J. Saad, Licio A. Velloso.

Artigo em vias de submissão

"Topiramate improves insulin and leptin signaling in hypothalamus and reduces obesity" a ser submetido ao Endocrinology. Autores: Caricilli AM, Penteado E, Mittestainer F, de Abreu LL, Quaresma P, Santos A, Guadagnini D, Razolli D, Velloso LA, Saad MJA, Prada PO.