

**UNIVERSIDADE DE SÃO PAULO**  
**INSTITUTO DE CIENCIAS BIOMÉDICAS**

Jéssica Aparecida da Silva Pereira

**RECEPTORES LXR E SEU PAPEL NA MODULAÇÃO DO METABOLISMO E  
FUNÇÃO DE MACRÓFAGOS RESIDENTES DO TECIDO ADIPOSEO: UMA NOVA  
FERRAMENTA PARA O TRATAMENTO DE DOENÇAS METABÓLICAS**

São Paulo, SP

2021

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Tese apresentada ao Programa de Pós  
Graduação em Imunologia como parte dos  
requisitos para obtenção do título de Doutor  
em Ciências.

Área de concentração: Imunologia

Orientador: Pedro M. M. de Moraes Vieira

São Paulo, SP

2021

Pereira, Jéssica Aparecida da Silva

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Orientador: Pedro Manoel Mendes de Moraes Vieira

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**UNIVERSIDADE DE SÃO PAULO**  
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Candidato (a): Jéssica Aparecida da Silva Pereira  
Título da tese: Receptores LXR e seu papel na modulação do metabolismo e função de macrófagos residentes do tecido adiposo: uma nova ferramenta para o tratamento de doenças metabólicas  
Orientador (a): Pedro Manoel Mendes de Moraes Vieira

A Comissão Julgadora dos trabalhos de Defesa da Tese de Doutorado, em sessão pública realizada a ...../...../....., considerou

**( ) Aprovado(a)      ( ) Reprovado(a)**

Examinador (a): .....

Instituição: .....

Examinador (a): .....

Instituição: .....

Examinador (a): .....

Instituição: .....

Examinador (a): .....

Instituição: .....

Presidente: .....

Instituição: .....

## CERTIFICADO

Certificamos que o projeto intitulado "*Efeitos da ativação dos receptores LXR sobre parâmetros metabólicos sistêmicos e na modulação do metabolismo de macrófagos teciduais residentes do tecido adiposo*", registrado sob o protocolo nº **51/2017**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de *Pesquisa Científica*, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle e Experimentação Animal (CONCEA). Ante esta conformidade, o referido projeto foi avaliado e aprovado em **09/05/2017** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Instituto de Ciências Biomédicas da Universidade de São Paulo (CEUA-ICB/USP), outorgando esta licença de uso de animais com validade de **4 ano(s)** a partir da data de aprovação.

- Investigador Principal: **Dr.(a.) Pedro Manoel Mendes de Moraes Vieira**

- Departamento: *Imunologia*

- Membros da Equipe: *Jéssica Aparecida da Silva Pereira (Pós-graduando)*

Ao final do período outorgado por esta licença, o pesquisador responsável deverá encaminhar a esta comissão, até o último dia de validade da atual proposta, *relatório final* de acordo com a Resolução Normativa CONCEA nº 30/2016 - Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica (DBCA), conforme modelo constante no endereço eletrônico [www.icb.usp.br/ceua](http://www.icb.usp.br/ceua). Havendo interesse na renovação do projeto, a solicitação deverá ser protocolada pela Secretaria da CEUA-ICB/USP até o último dia de validade da atual proposta. Após esta data uma nova proposta deverá ser encaminhada.

## CERTIFICATE

We hereby certify that the project entitled "*Effects of LXR receptor activation on systemic metabolic parameters and on the metabolic modulation of resident tissue macrophages of adipose tissue*", protocol nº **51/2017**, which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human), for *Scientific Research Purposes*, is in accordance with the provisions of the Law nº 11.794 passed on October 8<sup>th</sup>, 2008, Decree nº 6899 passed on July 15<sup>th</sup>, 2009, and the rules issued by the National Council for Control and Animal Experimentation (CONCEA). According to this legislation, the project was evaluated and approved on **5/9/2017** by the ETHICS COMMITTEE ON ANIMAL USE, Institute of Biomedical Sciences, University of Sao Paulo (CEUA-ICB/USP), and the license for animal use is valid for **4 year(s)** from the date of approval.

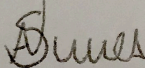
- Principal Investigator: **Dr.(a.) Pedro Manoel Mendes de Moraes Vieira**

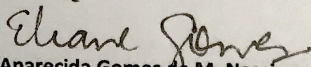
- Team members: *Jéssica Aparecida da Silva Pereira (Graduate Student)*.

At the end of the period granted by this license, the Principal Investigator must submit a final report of the project to this committee, according to the Rule nº 30 and the Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica (DBCA) issued by the CONCEA. If a renewal of the project is intended, the request must be submitted to the CEUA-ICB/USP secretary before the expiration of the current proposal. After this date, a new proposal must be prepared.

Espécie/Species	Linhagem/Strain	Sexo/Gender	Idade-Peso/ Age-Weight	Total
<i>Mus musculus</i>	C57BL/6	<i>Macho/male</i>	18-20 g	318
	C57BL/6 LXRaKO	<i>Macho/male</i>	18-20 g	78
	C57BL/6 LXRbKO	<i>Macho/male</i>	18-20 g	78

São Paulo, 11 de maio de 2017.

  
Prof. Dr. Anderson de Sá Nunes  
Coordenador CEUA-ICB/USP

  
Eliane Aparecida Gomes de M. Nascimento  
Secretário CEUA-ICB/USP



**CERTIFICADO**

Certificamos que a proposta intitulada Efeitos da ativação dos receptores LXR $\alpha$ s sobre parâmetros metabólicos sistêmicos e na modulação do metabolismo de macrófagos teciduais residentes do tecido adiposo, registrada com o nº 4562-1/2017, sob a responsabilidade de Prof. Dr. Pedro Vieira e Jéssica Aparecida da Silva Pereira, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008, que estabelece procedimentos para o uso científico de animais, do DECRETO Nº 6.899, DE 15 DE JULHO DE 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido aprovada pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP, em 22 de maio de 2017.

Finalidade:	( ) Ensino ( X ) Pesquisa Científica
Vigência do projeto:	15/05/2017-15/05/2021
Vigência da autorização para manipulação animal:	15/05/2017-15/05/2021
Espécie / linhagem/ raça:	Camundongo isogênico / C57BL/6J
No. de animais:	286
Peso / Idade:	05 semanas / 20g
Sexo:	246 machos / 40 fêmeas
Origem:	CEMIB/UNICAMP
Espécie / linhagem/ raça:	Camundongo Knockout / B6.129S6-Nr1h3 (LXR $\alpha$ KO)
No. de animais:	66
Peso / Idade:	05 semanas / 20g
Sexo:	machos
Espécie / linhagem/ raça:	Camundongo Knockout / B6.129S6-Nr1h2 (LXR $\beta$ KO)
No. de animais:	66
Peso / Idade:	05 semanas / 20g
Sexo:	machos
Espécie / linhagem/ raça:	Camundongo transgênico / B6.129P2-Lyztm1(cre)lfo/J
No. de animais:	04
Peso / Idade:	05 semanas / 20g
Sexo:	02 machos / 02 fêmeas
Espécie / linhagem/ raça:	Camundongo transgênico / B6.129S4(C) - VHLtm1jae/J
No. de animais:	04
Peso / Idade:	05 semanas / 20g
Sexo:	02 machos / 02 fêmeas
Origem:	The Jackson Laboratory

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao IBAMA, SISBIO ou CIBio e é restrita a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, 11 de maio de 2017.

\_\_\_\_\_  
 Profa. Dra. Liana Maria Cardoso Verinaud  
 Presidente

\_\_\_\_\_  
 Fátima Alonso  
 Secretária Executiva

**IMPORTANTE:** Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.

**CERTIFICADO**

Certificamos que a proposta intitulada **Efeitos da ativação dos receptores LXRs sobre parâmetros metabólicos sistêmicos e na modulação do metabolismo de macrófagos teciduais residentes do tecido adiposo**, registrada com o nº **4562-1(A)/2018**, sob a responsabilidade de **Prof. Dr. Pedro Vieira e Jéssica Aparecida da Silva Pereira**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA)**, tendo sido aprovada pela **Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP**, em reunião de **14/12/2018**.

Finalidade:	<input type="checkbox"/> Ensino <input checked="" type="checkbox"/> Pesquisa Científica
Vigência do projeto:	15/05/2017 a 15/05/2021
Vigência da autorização para manipulação animal:	14/12/2018 a 15/05/2021
Espécie / linhagem/ raça:	Camundongo isogênico / C57BL/6J
No. de animais:	30
Idade/Peso:	5.00 Semanas / 20.00 Gramas
Sexo:	30 Machos
Espécie / linhagem/ raça:	Camundongo Knockout / LXR duplo knock out
No. de animais:	30
Idade/Peso:	5.00 Semanas / 20.00 Gramas
Sexo:	30 Machos
Espécie / linhagem/ raça:	Camundongo isogênico / C57BL/6J
No. de animais:	30
Idade/Peso:	5.00 Semanas / 20.00 Gramas
Sexo:	30 Machos
Espécie / linhagem/ raça:	Camundongo Knockout / LXR duplo knock out
No. de animais:	30
Idade/Peso:	5.00 Semanas / 20.00 Gramas
Sexo:	30 Machos
Espécie / linhagem/ raça:	Camundongo Knockout / LXR duplo knock out
No. de animais:	30
Idade/Peso:	5.00 Semanas / 20.00 Gramas
Sexo:	30 Machos
Espécie / linhagem/ raça:	Camundongo isogênico / C57BL/6J
No. de animais:	30
Idade/Peso:	5.00 Semanas / 20.00 Gramas
Sexo:	30 Machos

**CERTIFICADO**

Certificamos que a proposta intitulada **Estudo dos efeitos da modulação dos fatores de transcrição LXR sobre o metabolismo sistêmico e inflamação do tecido adiposo em modelo de obesidade**, registrada com o nº **5205-1/2019**, sob a responsabilidade de **Prof. Dr. Pedro Manoel Mendes de Moraes Vieira e Jessica Aparecida da Silva Pereira**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido aprovada pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP, em reunião de **11/04/2019**.

Finalidade:	<input type="checkbox"/> Ensino <input checked="" type="checkbox"/> Pesquisa Científica
Vigência do projeto:	01/04/2019 a 01/04/2023
Vigência da autorização para manipulação animal:	11/04/2019 a 01/04/2023
Espécie / linhagem/ raça:	Camundongo isogênico / C57BL/6J
No. de animais:	33
Idade/Peso:	5.00 Semanas / 20.00 Gramas
Sexo:	33 Machos
Espécie / linhagem/ raça:	Camundongo isogênico / C57BL/6J
No. de animais:	33
Idade/Peso:	5.00 Dias / 20.00 Gramas
Sexo:	33 Machos
Espécie / linhagem/ raça:	Camundongo Knockout / LXRduploKO
No. de animais:	33
Idade/Peso:	5.00 Dias / 20.00 Gramas
Sexo:	33 Machos
Espécie / linhagem/ raça:	Camundongo Knockout / LXRduploKO
No. de animais:	33
Idade/Peso:	5.00 Dias / 20.00 Gramas
Sexo:	33 Machos
Origem:	Biotério, CEMIB, The Jackson Laboratory
Biotério onde serão mantidos os animais:	Biotério de Animais SPF, Área de Imunologia, DGF 1000 / UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização a junto ao IBAMA, SISBIO ou CIBio e é restrita a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.



*Ao meu companheiro de vida e de bancada, por todo apoio,  
amor e compreensão mesmo nos momentos mais difíceis*

## AGRADECIMENTOS

*Ao meu orientador e amigo Dr. Pedro M. M. Moraes Vieira, por ter me recebido em seu laboratório, pela total liberdade e confiança depositada em mim no desenvolvimento desse trabalho que sem sombra de dúvidas me deu a oportunidade de crescer profissionalmente.*

*Aos queridos João e Juliana, por serem excelentes alunos e grandes amigos. Vocês me incentivaram a sempre querer aprender mais e me mostraram o quão gratificante é ver a evolução profissional dos nossos pupilos. Espero ter atendido as expectativas de vocês.*

*Aos meus colegas de laboratório pelas discussões científicas e auxílio nos experimentos que contribuíram para o desenvolvimento desse trabalho.*

*Ao meu marido, companheiro e colaborador Gerson. Sou muito grata por nossa evolução pessoal e profissional e sinto muito orgulho do caminho que trilhamos juntos. Obrigada por ser tão incrível.*

*Aos amigos Henver e Tati que em tão pouco tempo de convívio se tornaram tão especiais. A conexão que temos é impressionante, eu simplesmente sinto que conheço vocês minha vida toda. Obrigada por serem amigos tão queridos. Vocês são família!*

*À minha grande amiga e profissional exemplar Elzira, você teve e tem um papel essencial na nossa jornada. Obrigada por ser essa grande amiga, que se preocupa, acolhe e cuida, mas que também dá broncas e puxões de orelha quando necessário. Amo você!*

*Aos queridos Felipe, João, Nati, André e André Santiago, com quem pude dividir momentos importantes profissionais e pessoais. Obrigada por todo carinho e apoio.*

*Às queridas Carol (Gremlin e tantos outros apelidos) e Lari por todas as experiências que compartilhamos durante nosso período em Boston.*

*Aos amigos Ju e Bozi, pessoas incríveis que me acolheram sempre. Muito obrigada pela amizade e incentivo profissional.*

*Ao Dr. Francisco Quintana e aos amigos do departamento de neurologia do BWH pelas discussões científicas e por toda ajuda durante o período em que estive em seu laboratório.*

*Aos amigos e agora padrinhos Fernando e Marisa por todo exemplo de integridade, pelo suporte incondicional e por sempre torcer pelo nosso sucesso*

*Aos meus familiares que sempre torceram pelo meu sucesso. Em especial aos queridos Joice e Wilian que nos deram amor e o apoio necessário em cada nova etapa dessa jornada. E ao Bailey, que chegou para completar nossa família.*

*Aos queridos Maria Eni e João, que sempre foram muito solícitos e ao Programa de Pós Graduação em Imunologia. Obrigada por toda ajuda ao longo desses anos.*

*Ao bioterista Marcos, que me auxiliou muito durante esse período e por seu trabalho essencial que possibilitou a realização de todos os experimentos desse trabalho.*

*Aos colaboradores que possibilitaram a realização de lindos experimentos que tornaram possível a realização desse trabalho, em especial ao Dr. Niels Olsen pelos reagentes, discussões e cafés, ao Dr. Alexandre Basso pela grande ajuda na aquisição dos animais e transplante de medula óssea e ao Dr. Marcelo Mori pelos reagentes, discussões e por me tornar uma quase integrante de seu laboratório.*

*Minha gratidão e respeito a todos os animais usados nesse trabalho, sem eles essa tese não existiria.*

*À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) que através de seu financiamento (processos 2017/00079-7 e 2019/04780-7) me proporcionou não somente a possibilidade de me dedicar a este trabalho, como também possibilitou a participação em diversos eventos e o network necessário para meu amadurecimento científico.*

*Às agências de fomento CAPES, CNPq e FUNCAMP pelo financiamento deste trabalho.*

*À todos que de algum modo participaram da realização deste trabalho, muito obrigada.*

*“Devo ater-me a meu próprio estilo e seguir meu próprio caminho. E apesar de eu poder nunca mais ter sucesso deste modo, estou convencida de que falharia totalmente de qualquer outro”*  
*Jane Austen*

## RESUMO

PEREIRA, JAS. **Receptores LXR e seu papel na modulação do metabolismo e função de macrófagos residentes do tecido adiposo: uma nova ferramenta para o tratamento de doenças metabólicas**. 2021. 88 f. Tese (Doutorado em Imunologia). São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2020.

Receptores hepáticos X (LXRs) são fatores de transcrição ativados por moléculas derivadas do metabolismo de colesterol. Os níveis de colesterol são frequentemente aumentados em indivíduos obesos e contribuem para a ativação de diversas vias inflamatórias. Obesidade é caracterizada pelo acúmulo de lipídeos nos adipócitos, favorecendo a expansão do tecido adiposo (AT) branco e o desenvolvimento de inflamação crônica sistêmica de baixo grau, a qual contribui para o desenvolvimento de outras doenças metabólicas, como a resistência à insulina. LXRs são envolvidos na manutenção da homeostase de colesterol, além disso, LXRs atuam na regulação das respostas imunes. No entanto, o papel de LXRs na patologia da obesidade e sua contribuição para o fenótipo de macrófagos residentes do tecido adiposo (ATM) permanece desconhecido. Nós mostramos que a deleção de LXR prejudica o ganho de peso e acúmulo de gordura em camundongos. Animais  $LXR\alpha\beta$ KO apresentam elevada sensibilidade à insulina e o uso preferencial de carboidratos como fonte de energia. Animais  $LXR\alpha$ KO, apresentam maior sensibilidade à insulina, o que não foi observado em animais  $LXR\beta$ KO. ATMs de animais obesos apresentam um enriquecimento da via de colesterol, e a deleção de LXR potencializa o acúmulo de lipídeos em macrófagos, bem como agrava a inflamação do AT por promover o aumento da expressão de IL-1 $\beta$  e TNF- $\alpha$  nos ATMs em resposta a alimentação com dieta hiperlipídica, o que ocorreu de maneira dependente de  $LXR\beta$ . Na ausência de estresse metabólico, a deleção de uma única isoforma de LXR foi capaz de induzir inflamação no AT, marcada pelo aumento de macrófagos CD11c<sup>+</sup>. Além disso, a deleção de LXR especificamente em células imunes contribuiu para o rompimento balanço imune no AT, o qual foi marcado pelo aumento de macrófagos CD11c<sup>+</sup>, pela maior expressão de IL-1 $\beta$ , bem como pelo maior infiltrado de monócitos. Em adição, a deleção de LXR em células imunes agravou a inflamação do AT induzida por dieta hiperlipídica, sem, no entanto, promover alterações na homeostase de glicose. Nós encontramos que o composto naringenin (NAR) atua como um agonista de LXR em macrófagos. NAR induziu a expressão de genes alvo de LXR e reduziu a secreção de citocinas pró-inflamatórias em macrófagos ativados com LPS ou polarizados para o perfil pró-inflamatório. O tratamento de animais obesos com NAR reduziu a inflamação do AT, marcada pelo menor acúmulo de macrófagos CD11c<sup>+</sup>, menor infiltrado de monócitos e reduzida expressão de IL-1 $\beta$ <sup>+</sup> e TNF- $\alpha$ <sup>+</sup> nos ATMs. Tal efeito favoreceu o aumento da sensibilidade à insulina nos animais tratados com NAR, o que não foi visto em animais tratados com veículo. Nós exploramos como a ativação de LXR com NAR poderia afetar o metabolismo de macrófagos, sendo observada redução da via glicolítica e menor disfunção mitocondrial em células estimuladas, tratadas com NAR. Adicionalmente, nós observamos que os efeitos anti-inflamatórios induzidos por NAR ocorrem de maneira dependente de  $LXR\beta$ . Juntos, nossos dados revelaram um papel diferenciado para as isoformas de LXR no que compete à regulação do metabolismo sistêmico e imunidade. A expressão de  $LXR\beta$  em células imunes é essencial para o balanço pro/anti-inflamatório no AT.  $LXR\beta$  é um potencial alvo terapêutico para o tratamento de inflamação do AT e resistência à insulina.

## SUMMARY

PEREIRA, JAS. **Liver X receptors and its role in macrophage metabolism and function: a new approach to treat metabolic diseases.** 2021. 88 f. Thesis (PhD in Immunology). São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2020.

Liver X receptors (LXRs) are nuclear receptors activated by molecules derived from cholesterol metabolism. Cholesterol levels are commonly increased in obesity and contribute for the activation of pro-inflammatory responses. Obesity is characterized by increased lipid accumulation in white adipocytes and systemic chronic low-grade inflammation. LXRs are involved in the maintenance of cholesterol homeostasis, and also have a role in the regulation of immune response. The role of LXRs in obesity and how these receptors contribute to adipose tissue macrophage (ATM) phenotype and metabolic programming remains to be elucidated. Here, we show that LXR deletion impairs body weight gain and fat accumulation in mice. LXR $\alpha$  $\beta$ KO mice has increased insulin sensitivity and a preferential usage of carbohydrates as fuel to energy production. LXR $\alpha$ -depleted but not LXR $\beta$ -depleted animals display increased insulin sensitivity with no changes in the energy fuel usage preference compared to WT mice. ATMs from obese animals have enriched cholesterol pathway, and LXR deletion boost the lipid accumulation in macrophages, while increase the adipose tissue inflammation by inducing IL-1 $\beta$  and TNF- $\alpha$  in ATMs in a LXR $\beta$ -dependent manner. In the absence of metabolic stress, the lack of any LXR isoform leads to adipose tissue inflammation with increased CD11c<sup>+</sup> ATMs. Also the LXR deletion specifically in immune cells contribute for the disruption of adipose tissue immune balance, marked by the increase in CD11c<sup>+</sup> and IL-1 $\beta$ <sup>+</sup> ATMs as enhanced monocyte infiltration on AT. Moreover, the deletion of LXR in immune cells aggravates obesity-induced adipose tissue inflammation without promote changes in glucose homeostasis. We found that naringenin (NAR) is an LXR agonist in macrophages. NAR induces LXR-target genes expression and reduces the secretion of pro-inflammatory cytokines in LPS-activated and LPS+IFN $\gamma$ -activated macrophages by reducing glycolysis and mitochondrial dysfunction. In addition, obese mice treated with NAR display increased insulin sensitivity and reduced AT inflammation, as observed by the reduction on CD11c<sup>+</sup>, IL-1 $\beta$ <sup>+</sup> and TNF- $\alpha$ <sup>+</sup> ATMs and reduced monocyte infiltration compared to obese control group. Together our results show that each LXR isoform displays a distinct role in the regulation of systemic glucose homeostasis and adipose tissue inflammation. LXR $\beta$  expression in immune cells is essential for the pro/anti-inflammatory balance of the adipose tissue. LXR $\beta$  has the potential to become a novel therapeutic target to treat adipose tissue inflammation and insulin resistance.

## LISTA DE ABREVIATURAS

- ABCA1 - *ATP-binding cassette sub-family A member 1*
- ABCG1 - *ATP-binding cassette sub-family G member 1*
- AF-1 - Domínio função de ativação-1
- AP1 - Proteína ativador 1, do inglês *activator protein 1*
- APOE - Apolipoproteína e
- ARG-1 - Arginase-1
- AT - Tecido adiposo
- ATMs - Macrófagos residentes do tecido adiposo, do inglês *adipose tissue macrophages*
- BAT - Tecido adiposo marrom, do inglês *brown adipose tissue*
- BMCs - Células da medula óssea, do inglês *bone marrow cells*
- BMDMs - Macrófagos derivados da medula óssea, do inglês *bone marrow-derived macrophages*
- BMT - Transplante de células da medula óssea, do inglês *bone marrow transplant*
- CCL-2/MCP1 - *Chemokine ligand 2/Monocyte chemoattractant protein-1*
- DBD - domínio de ligação ao DNA, do inglês *DNA-binding domain*
- DNA - Ácido deoxiribonucleico, do inglês *deoxyribonucleic acid*
- DRs - Sequências de repetições diretas
- DR-4 - Bases espaçadoras
- ECAR - Taxa de acidificação do meio extracelular, do inglês *extracellular acidification rate*
- ELISA - do inglês *Enzyme Linked ImmunonoSorbent Assay*
- FASN - Ácido graxo sintase, do inglês *fatty acid synthase*
- FIZZ1 - *Resistin-like molecule alpha 1*
- FXRs - Receptor ácidos biliares, do inglês *farnesoid X receptor*
- GLUT4 - Transportador de glicose tipo 4, do inglês *glucose transporter type 4*
- GTT - Teste de tolerância à glicose, do inglês *glucose tolerance test*
- HFD - Dieta hiperlipídica, do inglês *high fat diet*
- IFN- $\gamma$  - Interferon gama
- IL-1 $\beta$  - Interleucina 1 beta
- IL-12 - Interleucina 12

IL-4 - Interleucina 4  
IL-13 - Interleucina 4  
IL-10 - Interleucina 10  
IL-6 - Interleucina 6  
iNOS/NOS2 - Óxido nítrico sintase indutível, do inglês *inducible nitric oxide synthase*  
IRF - Fator regulatório de interferon 1, do inglês *interferon regulatory factor 1*  
IRS-1 - Substrato do receptor de insulina, do inglês *insulin receptor substrate-1*  
ITT - Teste de tolerância à insulina, do inglês *insulin tolerance test*  
LBD - Domínio de associação ao ligante, do inglês *ligand-binding domain*  
LDHA - Lactato desidrogenase isoforma a  
LPL - Lipoproteína lipase  
LPS - Lipopolissacarídeo  
LXR - Receptores hepáticos X, do inglês *liver X receptors*  
LXR $\alpha$  - Receptor hepático X isoforma alfa, do inglês *liver X receptor  $\alpha$*   
LXR $\beta$  - Receptor hepático X isoforma beta, do inglês *liver X receptor  $\beta$*   
LXR $\alpha$ KO - Camundongos deficientes de LXR $\alpha$   
LXR $\beta$ KO - Camundongos deficientes de LXR $\beta$   
LXREs - Elementos de resposta ao LXR  
M1 - Macrófagos classicamente ativados  
M2 - Macrófagos alternativamente ativados  
NAR - Naringenin  
NCoR - Co-repressor de receptores nucleares, do inglês *NR co-repressor*  
NF $\kappa$ B - Fator nuclear kappa B, do inglês *nuclear factor kappa B*  
NRs - Receptores nucleares, do inglês *nuclear receptors*  
Nr1h3 - gene codificador de LXR $\alpha$ , do inglês *nuclear receptor subfamily 1 group H member 3*  
Nr1h2 - gene codificador de LXR $\beta$ , do inglês *nuclear receptor subfamily 1 group H member 2*  
ORO - *Oil Red O*  
OXPHOS - Fosforilação oxidativa, do inglês *oxidative phosphorylation*  
PFKFB3 - 6-fosfofruto-2-quinase/frutose-2,6-bifosfatase  
PgAT - Tecido adiposo perigonadal



PPAR- $\gamma$  - Receptor ativado por proliferares de pejoxissoma gama, do inglês *peroxisome proliferator-activated receptor gamma*

REs - Elementos de resposta

RNA - Ácido ribonucleico, do inglês *ribonucleic acid*

RT-qPCR - Reação em cadeia da polimerase quantitativa em tempo real, do inglês *real time quantitative polymerase chain reaction*

RXR - Receptores de retinóide X, do inglês *retinoid X receptor*

SMRT - Mediador de silenciamento dos receptores retinóicos e de hormônio tireoidiano, do inglês *silencing mediator of retinoic acid and thyroid hormone receptors*

STAT6 - Tradutor de sinal e ativador de transcrição 6, do inglês *signal transducer and activator of transcription 6*

SVF - Fração vascular estromal, do inglês *stromal vascular fraction*

TCA - Ciclo do ácido cítrico, do inglês *tricarboxylic acid cycle*

TGF- $\beta$  - Fator de crescimento transformador beta, do inglês *transforming growth factor  $\beta$*

TLR4 - Receptor do tipo toll 4, do inglês *toll like receptor 4*

TNF- $\alpha$  - Fator de necrose tumoral alfa

VCO<sub>2</sub> - Volume de gás carbônico

VO<sub>2</sub> - Volume de oxigênio

WAT - Tecido adiposo branco, do inglês *white adipose tissue*

WT - Camundongos selvagens, do inglês *wild type*

YM1 - Chitinase-like protein 3

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## 1. INTRODUÇÃO

Obesidade é uma doença de etiologia complexa, marcada por um desequilíbrio energético causado pelo excessivo consumo alimentar associado a um gasto energético insuficiente (1, 2). Estima-se que cerca de 650 milhões indivíduos no mundo todo são obesos (3), o que faz com que a obesidade seja considerada uma das principais epidemias do século XXI (3, 4).

O tecido adiposo (AT) é um órgão amplamente distribuído pelo corpo humano (5), que apresenta um importante papel não somente de armazenamento, mas também na regulação de diversos processos metabólicos (6, 7). De maneira simplificada o tecido adiposo pode dividido em tecido adiposo marrom (BAT, do inglês *brown adipose tissue*) e tecido adiposo branco (WAT, do inglês *white adipose tissue*) (8).

O WAT é constituído por diversos tipos celulares, sendo os adipócitos a população majoritária, responsável por armazenar energia na forma de triacilgliceróis, proporcionar a disponibilidade de substratos energéticos, bem como pela secreção de adipocinas que contribuem de modo importante para a maturação de adipócitos, comunicação do status nutricional e na regulação do metabolismo de diversos outros tipos celulares (9-12). Além dos adipócitos maduros, o WAT é constituído por células da fração vascular estromal (SVF), a qual contém uma população celular heterogênea constituída por pré-adipócitos, fibroblastos e células imunes como macrófagos, eosinófilos e linfócitos T, dentre outros tipos celulares que desempenham um papel fundamental na regulação da homeostase tecidual (13, 14).

Alterações na demanda nutricional, como na obesidade, favorecem a expansão do WAT por promover o acúmulo excessivo de lipídeos (15), levando ao surgimento de uma inflamação crônica de baixo grau, caracterizada por alterações na população de macrófagos residentes do tecido adiposo (ATMs, do inglês *adipose tissue macrophages*) (4, 8, 16), bem como a um desequilíbrio imune caracterizado pela redução de citocinas anti-inflamatórias e pelo aumento na secreção de citocinas pró-inflamatórias como o fator de necrose tumoral alfa (TNF- $\alpha$ ) (17) e a interleucina 1 beta (IL-1 $\beta$ ), os quais tem sido fortemente associados ao desenvolvimento de resistência à insulina devido a sua capacidade em promover a redução da autofosforilação do receptor de insulina e subsequente redução da expressão e fosforilação do substrato do receptor de insulina (IRS-1, do inglês *insulin receptor substrate-1*) (18, 19).

Além disso, uma elevada concentração de ácidos graxos livres e colesterol é observada em indivíduos obesos, os quais podem promover alterações na expressão e ativação de diversos receptores nucleares que atuam como sensores de alterações metabólicas e controlam a programação transcricional, participando assim, na regulação do metabolismo e função celular (20).

### **1.1. Receptores Nucleares**

Receptores nucleares (NRs, do inglês *nuclear receptors*) correspondem a um dos maiores grupos de fatores de transcrição que desempenham papel fundamental na regulação de diversos processos fisiológicos, tais como desenvolvimento, reprodução, metabolismo e imunidade (21-23), e em muitos processos patológicos como câncer, diabetes e desordens metabólicas (24).

Os NRs são proteínas que regulam a expressão gênica através de sua associação a porções específicas do DNA, denominados elementos de resposta (REs), em decorrência de sua ativação mediada por ligantes (25). Ligantes de NRs geralmente correspondem à pequenas moléculas lipofílicas que transpõem facilmente as membranas biológicas, podendo variar entre hormônios e metabólitos tais como ácidos biliares, ácidos graxos e oxisteróis, dentre outras moléculas (26, 27).

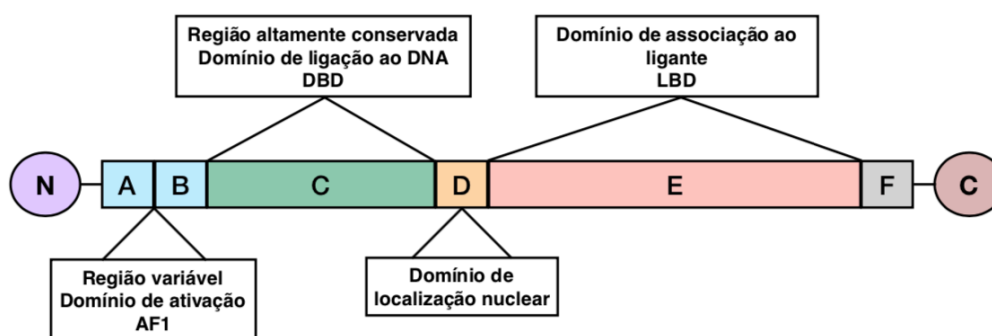
A associação de ligantes à NRs leva a numerosas modificações, destacando-se as alterações estruturais no receptor, que possibilitam sua ligação ao DNA e moléculas co-regulatórias essenciais para a regulação da programação transcricional (28). É importante destacar que embora a função melhor documentada dos NRs esteja associada a sua capacidade em regular a transcrição gênica, diversos trabalhos tem reportado que alguns NRs podem modular o funcionamento celular ainda no citoplasma (25).

De maneira geral, os NRs podem ser divididos em três principais grupos baseando-se em sua associação a ligantes (22). O primeiro grupo é constituído por NRs de esteroides, os quais são ativados mediante associação com hormônios esteróides, hormônios da tireóide e a algumas formas ativas de vitamina A e D (29, 30). O segundo grupo consiste em NRs órfãos, os quais não apresentam ligantes endógenos conhecidos (24, 31), e por fim, o terceiro grupo é constituído por NRs adotados, que correspondem a receptores previamente descritos e caracterizados como NRs órfãos e que, posteriormente, tiveram seu ligante endógeno

identificado (28). Dentre os receptores pertencentes ao grupo de NRs adotados destacam-se os PPARs, FXRs e LXRs (23, 32).

### 1.1.1. Características estruturais dos receptores nucleares

Assim como a maior parte dos NRs, os receptores hepáticos X (LXR, do inglês *liver X receptors*) apresentam domínios estruturais bem estabelecidos (28). De maneira simplificada, sua estrutura é composta por domínios A/B, C, D, E. O domínio A/B, também denominado domínio função de ativação-1 (AF-1) devido sua alta propriedade de ativação transcricional, compreende a um domínio amino-terminal altamente variado em tamanho e sequencia. O domínio central C consiste em uma porção altamente conservada que possibilita a associação do receptor ao DNA, denominado DBD (do inglês, *DNA-binding domain*). O domínio D corresponde a uma curta porção responsável pela localização nuclear. O domínio carboxi-terminal E ou LBD (do inglês, *ligand-binding domain*), é fundamental para a associação de ligantes e contribui para interações de receptores nucleares que formam heterodímeros. Alguns NRs possuem ainda um domínio F altamente variável próximo à porção carboxi-terminal, no entanto, a função deste domínio ainda não está bem estabelecida (Figura 1) (21).



**Figura 1** - Representação esquemática da composição estrutural dos receptores nucleares. NRs são constituídos por 6 domínios, os quais correspondem a uma porção N-terminal, um domínio de ativação (A/B), um domínio central de associação ao DNA (C), um domínio de localização nuclear (D), um domínio de associação ao ligante (E), e um domínio altamente variável (F) situado próximo à porção carboxi-terminal.

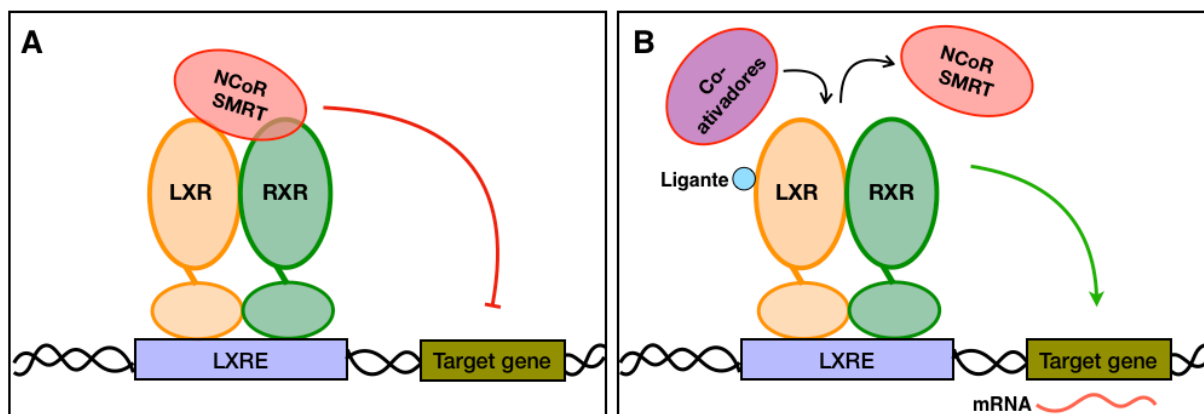
## 1.2. Receptores hepáticos X (LXR)

Os receptores hepáticos X (LXR, do inglês *liver X receptors*) são fatores de transcrição/receptores nucleares envolvidos na regulação de genes associados ao metabolismo de lipídeos e carboidratos (33-35). O modelo clássico proposto como mecanismo de ação de LXR baseia-se na formação de heterodímeros com receptores de retinóide X (RXR, do inglês *retinoid X receptor*), seguida da associação do complexo LXR-RXR a elementos de resposta



ao LXR (LXREs) (20). LXREs consistem em duas sequências de repetições diretas (DRs) de AGGTCA, separadas por quatro bases espaçadoras (DR-4) situadas em regiões promotoras de genes cuja transcrição é modulada por LXR (36).

A regulação da transcrição gênica promovida por LXR pode ocorrer de maneira dependente ou independente de ligantes (37). Na ausência de ligantes, a regulação transcricional ocorre através da formação de um complexo entre as moléculas co-repressoras NCoR (do ingles, *NR co-repressor*) e SMRT (do ingles, *silencing mediator of retinoic acid and thyroid hormone receptors*) com o heterodímero LXR-RXR que encontra-se ligado ao DNA, impedindo, dessa forma, a transcrição gênica (Figura 2A) (38). Ao passo que em resposta à associação com ligantes, a regulação gênica ocorre por meio de alterações conformacionais no receptor, as quais favorecem o desligamento das moléculas co-repressoras, e induz o recrutamento de moléculas co-ativadoras que facilitam a modificação das histonas e o remodelamento da cromatina, induzindo, desta forma, a transcrição gênica (Figura 2B) (24).



**Figura 2** - Representação esquemática do mecanismo de ação clássico dos receptores hepáticos X. **A** - Mecanismo inibitório independente de ligantes: Na ausência de ligantes moléculas co-repressoras impedem a transcrição gênica. **B** - Mecanismo de ativação dependente de ligantes: A associação de um ligante na porção LBD promove alterações estruturais no receptor que levam ao desligamento de moléculas co-inibitórias e subsequente aumento da afinidade por moléculas co-estimulatórias, resultando na ativação da transcrição gênica.

LXRs são ativados por moléculas oxidadas derivadas do metabolismo de colesterol, tais como oxisterol (37), e assim, atuam como sensores dos níveis de colesterol e exercem papel fundamental na regulação da transcrição de genes associados ao metabolismo, transporte e efluxo do colesterol (38).

Os receptores LXR apresentam duas isoformas, LXR $\alpha$  e LXR $\beta$ , codificadas pelos genes Nr1h3 e Nr1h2, respectivamente, que possuem distinto padrão de expressão e um alto grau de homologia (35, 39). A expressão de LXR $\alpha$  encontra-se restrita a órgãos com alta

atividade metabólica (35) e células da linhagem mielóide, como macrófagos (40), ao passo que a isoforma LXR $\beta$  encontra-se expressa de maneira constante, em todos os tipos celulares (39).

Além de sua importante participação na regulação do metabolismo de colesterol, LXRs desempenham um papel na manutenção da homeostase de glicose. De fato, dados da literatura mostram que LXRs podem ser ativados diretamente pela glicose ou seu derivados (41), bem como participar da regulação da expressão gênica de GLUT4 (42). Também tem sido reportado um importante papel para LXRs no que se refere a regulação das respostas imunes (43).

### **1.3. LXRs na regulação da função de macrófagos e inflamação do tecido adiposo**

Macrófagos são células da imunidade inata, altamente plásticas, que contribuem para a manutenção da homeostase em diversos processos biológicos (44). Em resposta a alterações em seu microambiente, essas células são capazes de apresentar um perfil de ativação diferenciado, o qual é frequentemente caracterizado por alterações em suas moléculas de superfície, bem como em seu perfil de secreção de citocinas, o que faz com que essas células exerçam uma participação direta na regulação de vias metabólicas distintas, bem como um grande envolvimento em diversas patologias (16, 45, 46).

Embora macrófagos apresentem um amplo espectro de fenótipos, de maneira simplificada essas células podem ser classificadas em dois subtipos opostos, caracterizados por sua capacidade em gerar uma resposta reparadora (M2) ou pró-inflamatórias (M1) (47-50).

Macrófagos classicamente ativados (M1), são induzidos *in vitro* em resposta ao estímulo pró-inflamatório mediado por LPS e IFN- $\gamma$  (51). *In vivo*, macrófagos M1 desempenham papel essencial na eliminação de patógenos, e são caracterizadas pela expressão de citocinas pró-inflamatórias como TNF $\alpha$ , IL-1 $\beta$  e IL-12, além de proteínas como a óxido nítrico sintase indutível (iNOS, do inglês *inducible nitric oxide synthase*) e no caso de macrófagos residentes do tecido adiposo (ATMs) a molécula de superfície CD11c (47, 52, 53).

A polarização de macrófagos alternativamente ativados (M2) *in vitro* ocorre em resposta ao estímulo com citocinas do padrão Th2 de resposta, como IL-4 e IL-13 (51). *In*



o fenótipo M1, caracterizado por duas quebras no TCA gerando o acúmulo de succinato e citrato, favorecendo a secreção de mediadores pró-inflamatórios e aumentado metabolismo glicolítico. A ativação de macrófagos com IL-4 e IL-13 induz o fenótipo M2, caracterizado pela secreção de mediadores anti-inflamatórios e pelo aumento da fosforilação oxidativa - OXPHOS (57).

Estudos têm reportado que alterações na população de macrófagos residentes do tecido adiposo (ATMs) e a produção excessiva de citocinas pró-inflamatórias estão diretamente relacionadas com o desenvolvimento de resistência à insulina, diabetes e aterosclerose em indivíduos obesos (48, 50, 62-64). Em condições de obesidade um acúmulo excessivo de macrófagos M1 no tecido adiposo é observado, os quais secretam citocinas pró-inflamatórias, como TNF- $\alpha$  e a quimiocina CCL-2 resultando na inibição da via da insulina, o que contribui para o quadro de síndrome metabólica (16, 17, 65). Nessa condição também se observa uma elevada concentração de ácidos graxos livres e colesterol, que podem promover alterações na expressão e ativação de LXRs e, conseqüentemente, no metabolismo e função dos ATMs (66).

Diversos trabalhos tem sido desenvolvidos com intuito de compreender a maneira pela qual LXRs contribuem para a ativação e função de células imunes (67-71). Dados na literatura mostram que o tratamento de células da imunidade inata com agonistas de LXR promovem um efeito anti-inflamatório devido sua capacidade em reprimir a expressão de genes pró-inflamatórios como *Nos2* e *Tnf- $\alpha$*  (72). Em resposta a um estímulo pró-inflamatório, fatores de transcrição como NF $\kappa$ B (do inglês, *nuclear factor kappa B*), AP1 (do inglês, *activator protein 1*) e IRF (do inglês, *interferon regulatory factor 1*) induzem a expressão de diversos genes pró-inflamatórios (73). Frente a sua associação com ligantes, LXRs são capazes de inibir expressão de genes pró-inflamatórios em macrófagos através da indução da expressão de genes associados ao efluxo de lipídeos em células imunes ou por um mecanismo de transrepressão de genes alvo de fatores de transcrição associados a resposta pró-inflamatória, a qual consiste na SUMOilação de LXR por SUMO2/3 (LXR $\alpha$ ) e SUMO1 (LXR $\beta$ ) e sua subsequente associação com NCoR, impedindo, desta forma, a associação de fatores de transcrição como NF $\kappa$ B, AP1 e IRF a região promotora de seus genes alvo (36, 73-79). Em adição a ativação de LXR promove maior expressão de marcadores anti-inflamatórios como a arginase I e arginase II, efeito não observados em macrófagos deficientes de LXR (80, 81), indicando que LXRs atuam como reguladores do elo entre resposta imune e metabolismo.

Embora muitos trabalhos tenham discutido as ações anti-inflamatórias de LXRs e seu papel na regulação do metabolismo lipídico, a maneira pela qual estes receptores modulam a função e o metabolismo de macrófagos *in vivo* ainda é pouco conhecida e estudos abrangendo a regulação do metabolismo de ATMs pelos LXRs e seu papel na resistência à insulina induzida pela obesidade ainda são escassos. Desta forma, temos como objetivo determinar como LXRs modulam a função de macrófagos residentes do tecido adiposo bem como sua contribuição para o desenvolvimento do quadro inflamatório e resistência à insulina induzida pela obesidade. Assim, nossa hipótese é que a ativação de LXRs irá inibir a ativação de macrófagos e a polarização para um perfil M1, sem alterar ou mesmo favorecendo a polarização para um perfil M2, resultando em menor inflamação no tecido adiposo e na consequente melhora da sensibilidade à insulina e da síndrome metabólica. Ainda, nós acreditamos que a ativação de LXR possa promover alterações no metabolismo celular e na função de macrófagos, levando estas células a um perfil menos glicolítico bem como a uma menor secreção de citocinas pró-inflamatórias.

## 2. JUSTIFICATIVA

Obesidade é uma doença crônica multifatorial, que acomete cerca de 600 milhões de pessoas no mundo todo sendo observado um aumento considerável no número de indivíduos com sobrepeso nos últimos anos. Estima-se que até o ano de 2025 cerca de 2,3 bilhões de pessoas estejam acima do peso, dentre as quais 700 milhões serão obesas (3). Associada com a resistência à insulina, a obesidade contribui para o desenvolvimento de outras desordens como dislipidemias, doenças cardiovasculares e diabetes mellitus tipo II, sendo considerada um dos principais fatores de risco para o desenvolvimento de síndrome metabólica (3, 82). Essa junção de transtornos cardiometabólicos acomete mais da metade da população de indivíduos adultos, sendo uma das principais causas de morbidade e mortalidade nos países desenvolvidos, tornando-se assim um dos grandes problemas de saúde pública da atualidade (83, 84).

Estudos têm reportado que alterações na população de macrófagos residentes do tecido adiposo e a produção excessiva de citocinas pró-inflamatórias estão diretamente relacionadas com o desenvolvimento de resistência à insulina, diabetes e aterosclerose em indivíduos obesos (16, 17, 65). Nesta condição também se observa uma elevada concentração de ácidos graxos livres e colesterol, que podem promover alterações na expressão de LXR<sub>s</sub> e conseqüentemente no metabolismo e função dos ATMs (62). Em adição, a administração de agonistas sintéticos de LXR<sub>s</sub> promovem melhora no quadro de resistência à insulina e intolerância a glicose em diversos tipos celulares, suportando a hipótese de que a modulação de LXR em macrófagos exerce grande participação no desenvolvimento de doenças metabólicas (62). Contudo, a maneira pela qual alterações metabólicas sistêmicas modulam a função e o metabolismo de macrófagos e como a ativação de LXR<sub>s</sub> em ATMs contribuem para o desenvolvimento de resistência a insulina ainda é pouco conhecida. Baseado nisso, nós hipotetizamos que a ativação de LXR em macrófagos irá inibir a ativação e a polarização para um perfil M1, resultando em menor inflamação do tecido adiposo e na conseqüente melhora na sensibilidade à insulina e síndrome metabólica

### **3. OBJETIVOS**

#### **3.1. Geral**

Analisar como os fatores de transcrição LXR modulam a função e o metabolismo de macrófagos.

#### **3.2. Específicos**

##### ***3.2.1. Determinar os efeitos da ativação de LXRs na polarização de macrófagos;***

*3.2.1.1. Avaliar a resposta de macrófagos frente a ativação de LXR in vitro;*

*3.2.1.2. Determinar os efeitos da ativação de LXR sobre a polarização (M{LPS+IFN $\gamma$ } ou M{IL-4}) e ativação (M{LPS}) de macrófagos;*

*3.2.1.3. Encontrar novos moduladores de LXR em macrófagos para subseqüente aplicação in vivo;*

*3.2.1.4. Determinar os efeitos da ativação de LXRs in vivo, avaliando parâmetros metabólicos sistêmicos bem como a modulação da polarização de ATMs de animais obesos;*

##### ***3.2.2. Determinar os efeitos da deleção (knockout) de Lxr sobre a polarização de macrófagos;***

*3.2.2.1. Avaliar o impacto da deleção de Lxr $\alpha$ , Lxr $\beta$  ou de ambos sobre perfil de ativação de macrófagos residentes do tecido adiposo (F4/80<sup>+</sup> CD11b<sup>+</sup> CD11c<sup>+</sup> e F4/80<sup>+</sup> CD11b<sup>+</sup> CD206<sup>+</sup>) de animais magros e obesos;*

*3.2.2.2. Investigar os efeitos da deleção de LXR sobre parâmetros metabólicos sistêmicos em animais submetidos ao modelo de obesidade;*

*3.2.2.3. Avaliar os efeitos da deleção de LXR especificamente em células imunes sobre a inflamação do tecido adiposo em animais magros e obesos.*

## 4. MATERIAIS E MÉTODOS

### 4.1. Animais

Para realização dos experimentos, foram empregados animais machos, adultos pesando cerca de 20 g, com idade aproximada de 5 semanas, das linhagens C57BL/6J (WT), B6.129S6-Nr1h3tm1Djm/J (LXR $\alpha$ KO) e B6.129S6-Nr1h2tm1Djm/J (Lxr $\beta$ KO). Animais WT foram fornecidos pelo Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório (CEMIB/UNICAMP), e animais LXR $\alpha$ KO e Lxr $\beta$ KO foram adquiridos da Jackson Laboratory. Animais LXR $\alpha\beta$ KO foram originados a partir do cruzamento das linhagens de animais LXR $\alpha$ KO e Lxr $\beta$ KO, empregando nos estudos apenas animais genotipados que apresentaram homozigose para ambas isoformas.

Para indução de obesidade, os animais foram alimentados ração hiperlipídica Envigo TD.93075, a qual apresenta teor calórico de 4,8 kcal/g, dos quais 55% são provenientes de gordura. O grupo não obeso foi alimentado com ração controle Nuvilab CR1, a qual apresenta 3,8 kcal/g, sendo 10% das calorias totais provenientes de gordura. Os animais foram monitorados durante o período de 14 a 16 semanas, e o ganho de peso e consumo alimentar avaliados semanalmente.

O tratamento com o agonista de LXR, naringenin (cat num. 14173 - Cayman Chemical), foi iniciado após confirmação do quadro de resistência à insulina em animais obesos, sendo administrados 10 mg/kg da droga pela via intraperitoneal. A administração de naringenin foi realizada diariamente durante 4 semanas, seguida da avaliação da sensibilidade à insulina. Como controle, animais magros e obesos foram tratados com o volume equivalente de veículo, o qual é composto por 50% PEG 400 (Sigma), 0,5% de Tween 80 (Sigma) e 49,5% de água destilada estéril. Após eutanásia dos animais, foi realizada coleta de soro para mensuração dos níveis de colesterol e triglicerídeos, bem como obtenção de diferentes tecidos para avaliação dos efeitos promovidos pela obesidade.

Todos os experimentos empregando animais foram aprovados pela CEUA: CEUA/UNICAMP no 4562-1/2017, 4562-1A/2018 e CEUA-ICB/USP no 51/2017. Os animais foram mantidos no biotério de experimentação animal do departamento de Imunologia, situado no Bloco F do Instituto de Biologia da UNICAMP, sob as condições de temperatura entre 20-22  $\pm$  2°C e umidade de 75-80%, tendo ciclo de luz/escuro de 12 horas durante todo o período de experimentação.



#### **4.2. Teste de Tolerância à Glicose e Insulina**

A avaliação da sensibilidade à insulina (ITT, do inglês *insulin tolerance test*) e tolerância à glicose (GTT, do inglês *glucose tolerance test*) foi realizada através da mensuração dos níveis de glicose presente na corrente sanguínea dos animais. Para realização do ITT, os animais foram submetidos a 5 horas de privação alimentar, e a glicemia foi avaliada, sendo este o tempo zero, seguida da administração intraperitoneal de 0,75 U/kg de insulina humulin R (Eli Lilly). Para realização do GTT, os animais foram submetidos a 12 horas de jejum, e os níveis glicêmicos foram avaliados para obtenção do tempo zero. Em seguida os animais foram submetidos a administração intraperitoneal de 1g/kg de D-Glucose (cat. num. G8270 - Sigma). O acompanhamento das alterações nos níveis glicêmicos em resposta aos diferentes estímulos foi realizado 15, 30, 60, 90 e 120 minutos após a injeção intraperitoneal de insulina ou glicose.

#### **4.3. Avaliação metabólica**

A avaliação do metabolismo dos animais foi realizada através de calorimetria indireta em circuito aberto. Para isso, animais WT, Lx $\alpha$ KO, Lx $\beta$ KO ou Lx $\alpha\beta$ KO foram mantidos em gaiolas metabólicas individuais no Oxymax/CLAMS (Columbus Instruments) durante 48 horas com ração hiperlipídica e água *ad libitum*. Foi realizado um período de aclimatação de 24 horas seguido de um período de medida da atividade metabólica (VO<sub>2</sub>, VCO<sub>2</sub>) por 24 horas, mantendo-se um ciclo de luz de 12h de maneira a determinar a atividade dos mesmos nos ciclos claro e escuro.

#### **4.4. Mensuração da gordura corporal**

A mensuração da composição corporal dos animais foi avaliada através do *Dual-energy X-ray absorptiometry* (DEXA scan) *In Vivo* Imaging System FX PRO (Carestream) situado no Centro de Facilidades para a Pesquisa (CEFAP) do Instituto de Ciências Biomédicas da USP. Para realização do procedimento, os animais foram anestesiados com solução composta por 100 mg/kg de quetamina e 10 mg/kg de xilazina, administrada por via intraperitoneal. Os animais anestesiados foram então posicionados no scanner de maneira ventral, com os membros e a cauda afastados do corpo. Posteriormente, foram adquiridas

imagens empregando dois níveis de energia de modo a possibilitar a avaliação do conteúdo de gordura corporal presente na região de interesse (ROI).

#### **4.5. Transferência adotiva de células imunes**

A deleção de LXR especificamente em células imunes foi obtida pela irradiação e subsequente transplante de células da medula óssea deficientes de LXR em animais WT seguindo o seguinte protocolo:

##### ***4.5.1. Irradiação***

Animais "aceptores" do transplante de medula óssea, entre 6 a 8 semanas de idade, foram acondicionados em um container de acrílico com fluxo de ar contínuo (28 cm diâmetro x 8 cm profundidade), situado entre duas fontes de raios  $\gamma$ . Os animais foram expostos a sessões de irradiação de modo a depletar todas as células imunes.

##### ***4.5.2. Transplante de medula óssea***

Células da medula óssea (BMCs) foram coletadas das tíbias e fêmurs de animais doadores LXR $\alpha\beta$ KO e WT, entre 6 a 8 semanas de idade. BMCs foram ressuspendidas em PBS acrescido de 2% de soro fetal bovino inativado (Gibco®). Um dia após a irradiação, os animais aceptores receberam injeção intravenosa pela veia caudal, utilizando uma agulha de 27 gauge, contendo  $3 \times 10^6$  BMCs provenientes dos doadores, sendo as combinações doador/acceptor LXR $\alpha\beta$ KO $\rightarrow$ WT e WT $\rightarrow$ WT. A fim de prevenir possíveis infecções, os animais foram tratados com sulfametoxazol + trimetoprima por via oral durante 7 dias e a repopulação das células imunes foi avaliada após 7 semanas do transplante. Após confirmada a eficácia do transplante, os animais foram submetidos a dieta hiperlipídica ou normolipídica durante 16 semanas e posteriormente a resistência à insulina e inflamação do tecido adiposo foram avaliados conforme descrito.

#### **4.6. Cultura de Células**

##### ***4.6.1. Macrófagos - Bone marrow-derived macrophages (BMDMs)***

Células da medula óssea proveniente do fêmur e tíbia de camundongos C57BL/6J wild type (WT), LXR $\alpha$ KO, Lxr $\beta$ KO e LXR $\alpha\beta$ KO foram obtidas e submetidas à lise de hemácias e

posterior centrifugação. O pellet celular foi ressuspensionado em 1 mL de meio RPMI e a contagem celular foi realizada. Após determinação do número de células obtido,  $1 \times 10^6$  células/poço foram adicionadas em placas de 12 poços em meio RPMI 1640 contendo 10% de soro fetal bovino (FBS), 1% de penicilina/streptomicina (P/S), 1% de aminoácidos não essenciais, 1% de piruvato de sódio, 1% de glutamina, 1% de Minimum Essential Medium, 0,125% de  $\beta$ -mercaptoetanol e 5% de sobrenadante proveniente do cultivo de células da linhagem L929. As células foram cultivadas durante 7 dias, sendo a troca de meio realizada no dia 5 e o estímulo com os moduladores de LXR, naringenin ou GW3965 (cat num.10054 - Cayman Chemical) e LPS ou a polarização com LPS+IFN $\gamma$  ou IL-4 realizada no dia 6 após o plaqueamento. A confirmação do perfil celular foi realizada através de citometria de fluxo, pela marcação de F4/80 e CD11b. E os efeitos da ativação de LXR sobre a função e metabolismo de macrófagos avaliados 24 horas após o estímulo.

#### **4.6.2. Adipócitos**

Após obtenção do tecido adiposo perigonadal de animais WT e LXR $\alpha\beta$ KO realizou-se a obtenção de células presentes na fração vascular estromal através da digestão tecidual com solução constituída por 2 mg/mL de colagenase II (Sigma) em meio RPMI1640 contendo 3,5% de FBS durante 1 hora. Após completa digestão, foi adicionado ao tubo contendo a suspensão celular 10% de FBS para inativação da colagenase. A suspensão foi centrifugada a 1200 rpm, a 4°C durante 10 minutos. O sobrenadante contendo adipócitos maduros foi descartado e ao pellet celular contendo a fração vascular estromal adicionou-se 2 mL de tampão para promover a lise de hemácias, seguindo com uma nova etapa de centrifugação. O pellet celular foi ressuspensionado em 1 mL de meio DMEM e a contagem celular foi realizada. Após determinação do número de células obtido,  $1 \times 10^6$  células/poço foram adicionadas em placas de 6 poços em meio de crescimento constituído por DMEM high glucose, 10% FBS, 1% Ciprofloxacino a 1 mg/mL, 0,1% de biotina a 33 mM (Sigma) e 0,1% de ácido pantotênico a 17 mM (Sigma). As células foram mantidas em estufa a 37°C com 5% de CO $_2$ , sendo a troca de meio realizada no dia seguinte ao plaqueamento, para remoção de artefatos. Posteriormente, o meio de cultivo foi trocado a cada 2 dias até que as células entrarem em confluência. A diferenciação das células provenientes da fração vascular estromal foi realizada empregando o meio de indução composto por DMEM high glucose, 10% FBS, 1%

Ciprofloxacino a 1 mg/mL, 0,1% de biotina a 33 mM (Sigma) e 0,1% de ácido pantotênico a 17 mM (Sigma), rosiglitazona 1  $\mu$ M, insulina 850 nM, IBMX 250  $\mu$ M e dexametasona 1  $\mu$ M, o qual foi empregado por 3 dias, e posteriormente pelo emprego do meio de diferenciação composto por DMEM high glucose, 10% FBS, 1% Ciprofloxacino a 1 mg/mL, 0,1% de biotina a 33 mM (Sigma) e 0,1% de ácido pantotênico a 17 mM (Sigma) e rosiglitazona 1  $\mu$ M, o qual foi substituído a cada 2 dias durante o período de 15 dias. Após completa diferenciação dos adipócitos brancos, as células foram lavadas com PBS e mantidas em meio sem FBS durante 8 horas, e posteriormente estimuladas com 100 nM de insulina durante 15 minutos. Após esse período, as células foram lavadas com PBS, coletadas e empregadas na avaliação da fosforilação de Akt por western blot ou na avaliação da expressão genes envolvidos na adipogênese por qPCR.

#### **4.7. Citometria de Fluxo**

Alterações no potencial de membrana mitocondrial de macrófagos foram avaliadas por citometria de fluxo, empregando os marcadores MitoTracker™ Green (M7514 - Invitrogen™) FM e MitoTracker™ Red CMXRos (M7512 - Invitrogen™). Para os experimentos *in vivo*, a distinção da população de leucócitos presentes no tecido adiposo visceral dos animais foi realizada através da marcação de CD45 (Pacific Blue - clone 30-F11), F4/80 (FITC - clone BM8), CD11b (APC-Cy7 - clone M1/70), CD206 (APC - clone C069C2), CD11c (BV421 - clone N418), Ly6C (PE-Cy7 - clone HK1.4), Ly6G (PercP - clone 1A8) e Siglec F (PE - clone E50-2440) (BD Pharmingen™) e pela avaliação do tamanho e complexidade de cada tipo celular. Em todos os casos foi realizada marcação com Zombie Aqua™ Fixable Viability Kit (Biolegend) para exclusão de células mortas nas análises posteriores, bem como o emprego de células não marcadas como controle. Para avaliação da expressão de citocinas, as células foram fixadas com Cytifix® (BD Biosciences) após a marcação das moléculas de superfície e permeabilizadas com o PermWash® (BD Biosciences). A marcação intracelular foi realizada para as citocinas IL-12 (APC - clone C15.6), IL-1 $\beta$  (PE - clone NJTEN3) e TNF- $\alpha$  (PE-Cy7 - clone MP6-XT22). Os eventos foram adquiridos em citômetro de fluxo BD FACSVersé (BD Biosciences) após compensação apropriada das cores. Todas as aquisições foram analisadas através do software *FlowJo*®.

#### **4.8. Quantificação de citocinas secretadas**

Possíveis alterações no perfil de citocinas secretadas por BMDMs foram verificadas através da técnica de ELISA (*Enzyme Linked Immunosorbent Assay*), utilizando amostras do sobrenadante proveniente da cultura celular. As dosagens foram realizadas utilizando os kits para quantificação de TNF- $\alpha$  (Biolegend), IL-6 (BD), IL-12 (BD), IL-1 $\beta$  (Biolegend), MCP-1 (BD) e IL-10 (BD) segundo instruções do fabricante. As amostras foram diluídas 10 vezes para a dosagem de TNF- $\alpha$  e IL-12, 20 vezes para IL-6 e MCP-1, 2 vezes para IL-10 e amostras não diluídas para quantificação de IL-1 $\beta$ . A leitura das absorbâncias foi realizada em espectrofotômetro SPECTRA max M3 (Molecular Devices) em comprimento de onda de 450 nm com correção em 570 nm.

#### **4.9. Extração de RNA e Síntese de cDNA**

A expressão dos genes alvo de LXR e genes associados ao metabolismo e ativação de macrófagos em BMDMs e no tecido adiposo foi analisada por RT-qPCR. Para realização destas análises, o RNA total foi extraído utilizando o reagente a base de fenol TRI Reagent® (Sigma-Aldrich). As concentrações do RNA obtido foram avaliadas através do uso de Nanodrop® (Thermo Scientific) apresentando uma relação 260 nm /230 nm e 260 nm /280 nm próxima de 2. Após a extração e quantificação do RNA foi realizada a síntese de cDNA empregando o kit GoScript™ Reverse Transcription Mix (Oligo dT) (Promega) de acordo com as instruções do fabricante e o cDNA obtido empregado em análises de expressão gênica.

#### **4.10. Análises de expressão gênica por PCR quantitativo**

As reações de RT-qPCR foram realizadas empregando SYBR® Green Supermix (Biorad). Para cada reação de qPCR foram utilizados 3  $\mu$ L de SYBR® Green Supermix 2x, 0,25  $\mu$ L de iniciador forward 10  $\mu$ M, 0,25  $\mu$ L de iniciador reverse 10  $\mu$ M e 3  $\mu$ L de cDNA 10 ng/ $\mu$ L. Após incubação por 10 minutos a 95°C, a amostra foi submetida a 40 ciclos de 20s a 95°C, 30s a 95°C e 30s a 60°C. Os valores de  $\Delta\Delta$ CT obtidos foram utilizados para quantificação da expressão de cada gene, sendo empregados como normalizadores o gene ribossomal 18S e como controle negativo a ausência de cDNA. A sequencia dos primers empregados nos ensaios e na genotipagem dos animais encontra-se disponível na tabela 01.

**Tabela 1** - Iniciadores utilizados para análise do padrão de expressão gênica de marcadores de ativação de macrófagos e genes alvo de LXR.

Descrição		Sequência 5' - 3'
<i>Retnlα</i> (Fizz1)	<i>Foward</i>	TACTTGCAACTGCCTGTGCTTACT
<i>Retnlα</i> (Fizz1)	<i>Reverse</i>	TATCAAAGCTGGGTTCTCCACCTC
<i>Chil3</i> (Ym1)	<i>Foward</i>	TCTCTACTCCTCAGAACCGTCAGA
<i>Chil3</i> (Ym1)	<i>Reverse</i>	GATGTTTGTCTTAGGAGGGCTTC
<i>Clec10α</i> (Mgl1/CD301)	<i>Foward</i>	TGAGAAAGGCTTTAAGAACTGGG
<i>Clec10α</i> (Mgl1/CD301)	<i>Reverse</i>	GACCACCTGTAGTGATGTGGG
<i>Tnf-α</i>	<i>Foward</i>	TAGCCCACGTCGTAGCAAAC
<i>Tnf-α</i>	<i>Reverse</i>	TGTCTTTGAGATCCATGCCGT
<i>Il-1β</i>	<i>Foward</i>	GGCAGCTACCTGTGTGTCTTTCCC
<i>Il-1β</i>	<i>Reverse</i>	ATATGGGTCCGACAGCACGAG
<i>Nos2</i>	<i>Foward</i>	TGGTGAAGGGACTGAGCTGT
<i>Nos3</i>	<i>Reverse</i>	CCAACGTTCTCCGTTCTCTTG
<i>Pfkfb3</i>	<i>Foward</i>	GTCGCCGAATACAGCTACGA
<i>Pfkfb3</i>	<i>Reverse</i>	CCCACAGGATCTGGGC
<i>Abca1</i>	<i>Foward</i>	AACAGTTTGTGGCCCTTTTG
<i>Abca1</i>	<i>Reverse</i>	AGTTCCAGGCTGGGGTACTT
<i>Abcg1</i>	<i>Foward</i>	GGTCCTGACACATCTGCGAA
<i>Abcg1</i>	<i>Reverse</i>	CAGGACCTTCTTGGCTTCGT
<i>Lpl</i>	<i>Foward</i>	TGCCCCGAGGTTTCCACAAAT
<i>Lpl</i>	<i>Reverse</i>	GCCAGCTGAAGTAGGAGTCG
<i>Fasn</i>	<i>Foward</i>	GCATAACGGTCTCTGGTGGT
<i>Fasn</i>	<i>Reverse</i>	CGAACTTTTCCAAGGTGGGG
<i>Apoe</i>	<i>Foward</i>	ACAGATCAGCTCGAGTGGCAA
<i>Apoe</i>	<i>Reverse</i>	ATCTTGCGCAGGTGTGTGGAGA
<i>Nr1h3</i> (LXRα)	<i>Foward</i>	TCAGTGGAGGGAAGGAAATG
<i>Nr1h3</i> WT (LXRα)	<i>Reverse</i>	TTCCTGCCCTGGACACTTAC

<i>Nr1h3</i> Mutante (LXR $\alpha$ )	<i>Reverse</i>	TTGTGCCCAGTCATAGCCGAAT
<i>Nr1h2</i> (LXR $\beta$ )	<i>Foward</i>	CTGTCTTTTGGTCCGCTTTC
<i>Nr1h2</i> WT (LXR $\beta$ )	<i>Reverse</i>	CACCCCTTGCTTACTACTGCT
<i>Nr1h2</i> Mutante (LXR $\beta$ )	<i>Reverse</i>	CCAGTCATAGCCGAATAGCC
<i>18S</i>	<i>Foward</i>	CTCAACACGGGAAACCTCAC
<i>18S</i>	<i>Reverse</i>	CGCTCCACCAACTAAGAACG

#### 4.11. Avaliação do metabolismo celular

A determinação dos efeitos da modulação de LXR sobre o metabolismo de macrófagos foi avaliada através do SeaHorse XFe24 Analyzer (Agilent). Para isso,  $0,5 \times 10^6$  células/poço foram cultivadas e diferenciadas na microplaca XF 24 compatíveis com o equipamento, empregando meio de cultura RPMI 1640 contendo 10% de soro fetal bovino, 1% de penicilina/streptomicina, 1% de aminoácidos não essenciais, 1% de piruvato de sódio, 1% de glutamina, 1% de Minimum Essential Medium, 0,125% de  $\beta$ -mercaptoetanol e 5% de sobrenadante proveniente do cultivo de células da linhagem L929. No sétimo dia de diferenciação, o meio de cultura das BMDMs foi substituído por meio DMEM (Agilent) suplementado com 25 mM de glicose (Sigma), 2 mM de piruvato (Sigma) e 1 mM de glutamina (Lonza) e mantidas por 1 hora em estufa a 37°C sem CO<sub>2</sub>. Nas 12 horas que antecederam a realização do experimento, o cartucho de sensores XF24-3 (Agilent) foi hidratado com solução calibrante XF (Agilent) e acondicionado em estufa a 37°C sem CO<sub>2</sub>. A calibração do equipamento foi realizada de acordo com instruções do fabricante. Cada cartucho possui 4 canais, permitindo a injeção de até 4 diferentes moduladores por poço. O canal 1 foi preenchido com 75  $\mu$ L de naringenin 3,84  $\mu$ M de modo a ficar em uma concentração final de 0,5  $\mu$ M na placa, após a injeção pelo equipamento. O canal 2 foi preenchido com 867 ng/mL de LPS, sendo a concentração final de LPS 100 ng/mL. Como controle os canais foram preenchidos com 75  $\mu$ L de PBS. Após a calibração, a placa contendo a solução calibrante foi substituída pela placa contendo as células. A injeção de naringenin ou PBS foi realizada após 30 minutos decorridos do início do experimento, e a adição de LPS ou PBS foi realizada 30 minutos após a injeção de naringenin. A avaliação da taxa de glicólise

foi acompanhada através do ECAR (taxa de acidificação do meio extracelular) durante 6 horas.

Para realização do MitoStress Test o canal 1 foi preenchido com 56  $\mu\text{L}$  de oligomicina 9,93  $\mu\text{M}$  de modo a ficar em uma concentração final de 1  $\mu\text{M}$  na placa, após a injeção pelo equipamento. O canal 2 foi preenchido com 62  $\mu\text{L}$  de FCCP 14,95  $\mu\text{M}$ , sendo a concentração final variando entre 1,5  $\mu\text{M}$ . O canal 3 foi preenchido com 69  $\mu\text{L}$  de solução composta de antimicina e rotenona, sendo a concentração final de 1  $\mu\text{M}$  de ambas as drogas. Após a calibração, a placa contendo a solução calibrante foi substituída pela placa contendo as células. A injeção de oligomicina foi realizada após 20 minutos decorridos do início do experimento, a adição de FCCP foi realizada após 45 minutos do início da corrida, e a última injeção, com antimicina/rotenona ocorreu após 70 minutos do início da corrida. A avaliação da respiração mitocondrial dos macrófagos foi acompanhada através do OCR (*oxygen consumption rate*), sendo o tempo total da corrida de 100 minutos.

#### **4.12. Marcação de corpúsculos lipídicos com Oil Red O**

Após os estímulos com LPS ou tratamento com moduladores de LXR, BMDMs e macrófagos peritoneais foram submetidos à marcação com Oil Red O para avaliação da presença de corpúsculos lipídicos no interior destas células. Para tanto, macrófagos foram fixados em formalina 3,7 % por 30 minutos e posteriormente corados com Oil Red O 60%. A visualização de lipídeos neutros foi efetuada através de microscópio óptico de campo claro e fluorescência, sendo neste caso, a marcação de núcleos realizada com DAPI.

#### **4.13. Western Blotting**

Após a lise celular e quantificação de proteínas através do kit Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), as amostras foram preparadas de modo a obter 40  $\mu\text{g}$  de proteínas totais e acrescidas de tampão de amostra, posteriormente amostras foram aquecidas a 95°C durante 5 minutos e aplicadas em gel de poliacrilamida a 12%, tendo como padrão o marcador de peso molecular TrueColor High Range Protein Marker (cat num S2600 - Sinapse Inc). A corrida foi realizada empregando 100V até que as amostras saíssem do gel de empilhamento e alcançassem o gel de separação, e então elevada para 120V até o termino da corrida. A transferência foi realizada em membrana de nitrocelulose Amersham Protran 0,45



NC (cat. num. 10600003 - GE Healthcare), em tampão Tris 25 mM, Glicina 190 mM, SDS 0,1% , a 200 mA, durante 4 horas em banho de gelo. Após transferência as membranas foram lavadas com tampão TBS-T e incubadas por 1 hora com solução de bloqueio constituída por leite desnatado 5% ou BSA 3% em diluídos em TBS-T.

Para as amostras de adipócitos, posterior a este período, a solução de bloqueio foi descartada e adicionou-se a membrana o anticorpo policlonal produzido em coelho anti-phospho-Akt (Ser473) (cat. num. 9271 - CellSignaling) na proporção 1:1000 diluído em solução de bloqueio, e seguiu-se sob incubação overnight a 4°C. Para as amostras de macrófagos, empregou-se anticorpo policlonal produzido em coelho anti-IkBa (cat. num. 9242 - CellSignaling) na proporção 1:1000 ou o anticorpo policlonal produzido em coelho anti-phospho-p38 MAPK (Thr180/Tyr182) (cat. num. 9211 - CellSignaling) na proporção 1:1000. Ambos anticorpos foram diluídos em solução de bloqueio, e incubados overnight a 4°C. Posteriormente, a membrana foi submetida a 5 ciclos de lavagem com duração de 2 minutos em tampão TBS-T e adicionou-se anticorpo secundário Rabbit IgG HRP (cat. num. NA934V – GE Healthcare) na proporção 1:10000 diluído em solução de bloqueio, seguindo incubação a temperatura ambiente durante uma hora. Após este período a membrana foi lavada novamente e adicionou-se a esta o substrato quimioluminescente SuperSignal™ West Pico PLUS (Thermo Fisher Scientific). A membrana foi então revelada no sistema de imagem ChemiDoc™ Gel Imaging System (Biorad). Como controle a membrana foi incubada com o anticorpo policlonal produzido em coelho anti-Akt (cat. num. 9272 - CellSignaling) ou com o anticorpo monoclonal feito em camundongo anti- $\alpha$ -Tubulin (cat. num. T5168 - Sigma), na proporção 1:1000 pelo período de uma hora, para adipócitos e macrófagos, respectivamente. A revelação da membrana seguiu conforme descrito anteriormente.

A análise do nível de expressão das proteínas de interesse foi realizada através do programa Image J, empregando a densitometria das bandas apresentadas para as proteínas alvo e normalizadas pela expressão de  $\alpha$ -Tubulin ou Akt da amostra correspondente, sendo os valores expressos através da razão proteína de interesse/proteína controle.

#### **4.14. Análises estatísticas**

As análises estatísticas de todos os dados obtidos foram realizadas com o programa GraphPad Prism® versão 9.0, empregando oneway ANOVA para análise de variância e

*posthoc* de Bonferroni ou *t-test* para verificação da diferença em uma variável entre os grupos experimentais. O mesmo programa foi utilizado para construção dos gráficos.

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## **APENDICE 1**

## Review Article

# The Impact of Ghrelin in Metabolic Diseases: An Immune Perspective

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Obesity and insulin resistance have reached epidemic proportions. Obesogenic conditions are associated with increased risk for the development of other comorbidities and obesity-related diseases. In metabolic disorders, there is chronic low-grade inflammation induced by the activation of immune cells, especially in metabolic relevant organs such as white adipose tissue (WAT). These immune cells are regulated by environmental and systemic cues. Ghrelin is a peptide secreted mainly by X/A-like gastric cells and acts through the growth hormone secretagogue receptor (GHS-R). This receptor is broadly expressed in the central nervous system (CNS) and in several cell types, including immune cells. Studies show that ghrelin induces an orexigenic state, and there is increasing evidence implicating an immunoregulatory role for ghrelin. Ghrelin mainly acts on the innate and adaptive immune systems to suppress inflammation and induce an anti-inflammatory profile. In this review, we discuss the immunoregulatory roles of ghrelin, the mechanisms by which ghrelin acts and potential pharmacological applications for ghrelin in the treatment of obesity-associated inflammatory diseases, such as type 2 diabetes (T2D).

## 1. Introduction

The incidence of obesity and insulin resistance has increased in recent years. The World Health Organization (WHO) estimates that approximately 600 million adult people are obese [1]. Obesity directly impacts the economy and the quality of life of affected patients [2, 3]. Obesity is a disease with multifactorial origins and is characterized by excessive lipid accumulation in white adipose tissue (WAT), is promoted by the imbalance between caloric intake and energy expenditure [4, 5], and has harmful consequences to the individual [6]. Obesity is a risk factor for the development of other diseases, such as type 2 diabetes (T2D), metabolic syndrome, cardiovascular diseases, atherosclerosis, and several types of cancer [6–10].

WAT is a critical organ that contributes to host metabolism. Several cell types reside in WAT that regulate WAT and

systemic homeostasis, such as adipocytes and preadipocytes, fibroblasts, macrophages, T lymphocytes, and among several other immune and nonimmune cells [11–15]. During obesity, immune alterations are observed in response to WAT expansion, which lead to a low-grade chronic inflammation. This inflammatory response is induced by changes in the recruitment of new leukocytes and also by changes in the function and activation status of adipose tissue resident macrophages (ATMs) and other leukocytes [6, 16, 17]. The immune changes that occur in WAT are characterized by the reduction of anti-inflammatory cytokines, such as interleukin 10 (IL-10), and upregulation of proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). This leads to the inhibition of the insulin-signaling pathway, which results in systemic insulin resistance [17].

Disruption of adipose tissue (AT) homeostasis and the induction of chronic systemic inflammation caused by

obesity are complex processes and involve many players [16]. The disturbances within WAT microenvironment occur at the immune and metabolic levels in obesity and obesity-related conditions. Among these changes, increased levels of circulating free fatty acids (FFA) contribute to the development of insulin resistance [18]. Elevated levels of FFAs lead to the generation of new metabolites from FFA reesterification, such as diacylglycerol (DAG) [19]. DAG promotes the activation of several serine/threonine kinases, such as protein kinase C (PKC), which drastically impairs the phosphorylation of insulin receptor substrates (IRS) 1/2, thereby disrupting insulin signaling [18–20].

Another important players in the induction and control of AT inflammation are the Toll-like receptors (TLR), in particular, TLR4 [21, 22]. TLR activation leads to defective cellular function in all metabolically relevant organs, such as the liver, pancreas, and WAT [20–24]. This defect in cellular function results in immune cell activation and inflammation subsequently leading to resistance to key metabolic hormones such as insulin, leptin, and ghrelin [23–27].

Ghrelin is a peptide-hormone/cytokine widely distributed throughout the gastric mucosa made up of 28 amino acids and is mainly secreted by X/A-like enteroendocrine cells [28–31]. Ghrelin was described in 1999 as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [32], a G-coupled receptor broadly expressed in the central nervous system (CNS) and in peripheral tissues, including nerve cells, cardiac cells, adipocytes, and immune cells [32–34].

Ghrelin has an important role in obesity and metabolic-related disorders. It is most known for its role in appetite regulation, acting directly on hypothalamic neurons responsible that were involved in feeding behavior [35]. Beyond this “classic” function, ghrelin is also an immunomodulatory hormone, providing new perspectives for its relevance in metabolic diseases [36, 37]. In obesogenic conditions, ghrelin levels are reduced with a concomitant induction of chronic low-grade inflammation [23, 38]. These data strongly suggest a role for ghrelin in obesity-related pathological conditions in establishing and maintaining “metabolic inflammation” and expand our knowledge of ghrelin beyond its role in the CNS. In this review, we will discuss the participation of ghrelin in immunomodulatory events, the impact of this regulation on metabolic disorders, and the mechanisms by which ghrelin acts.

## 2. Ghrelin Structure, Function, and Receptor

The GHS-R has two isoforms, GHS-R1a and GHS-R1b [39]. Only GHS-R1a triggers a signaling pathway, which is induced by the binding of ghrelin [39]. The lack of GHS-R1b isoform activity is attributed to the absence of a third intracellular loop, which prevents G protein coupling [39, 40]. There is evidence describing the interaction between ghrelin receptor and other G-coupled receptors, such as dopamine, serotonin and melanocortin receptors, and even GHS-R1b [39, 41]. These interactions lead to conformational changes in GHS-R, which impact GHS-R1a signaling [39–41].

Secreted ghrelin is found in two distinct forms in the bloodstream [42]. One is the desacyl (desoctanoyl) form (desacyl-ghrelin), which is more stable and has higher serum concentration levels compared to other ghrelin form [43–45]. Desacyl-ghrelin is suggested to be a non-GHS-R1a ligand form of ghrelin under physiological conditions [31, 43, 45]. Desacyl-ghrelin has cardioprotective effects [46–48]. However, its functional role and the receptor by which desacyl-ghrelin binds remain unknown [39, 49]. The other form of ghrelin is the acylated form (acyl-ghrelin), which undergoes a posttranslational modification on serine residue 3 [50]. This acylated form corresponds to approximately 20% of total circulating ghrelin and is responsible for the biological effects of ghrelin [51] and indicates that acylation of ghrelin is an important step for the biological activity of this peptide [51–53].

The posttranslational structural modification observed in the acylated form of ghrelin is attributed to an enzyme, discovered in 2008 by Yang and colleagues [54], which is called ghrelin-O-acyltransferase (GOAT). GOAT is responsible for the acylation of the preproghrelin before it is transported to the Golgi apparatus [42]. In the Golgi vesicle, proghrelin is proteolytically cleaved by the prohormone convertase 1/3 (PC 1/3) [31, 55, 56].

Acyl-ghrelin has a wide range of functions in several tissues. Acyl-ghrelin stimulates growth hormone secretion by the pituitary gland and activates the hypothalamic orexigenic axis [57]. Ghrelin serum levels are increased during caloric restriction [30]. In the hypothalamic orexigenic axis, ghrelin induces the secretion of neuropeptides, such as AgRP (agouti-related protein) and NPY (neuropeptide Y) [58], which leads to increased food consumption and reduced energy expenditure [53].

In addition to the direct effects of ghrelin on the CNS, ghrelin regulates gastrointestinal motility [59, 60], energy homeostasis [61], and the cardiovascular and reproductive systems [62]. Ghrelin also participates in the regulation of other adipokines, such as leptin, and modulates a broad number of immune functions [31, 63].

## 3. Ghrelin Signaling

GHS-R1a is widely distributed in different tissues [32–34], and its activation by acyl-ghrelin involves several signaling pathways [28–30]. The most studied cell type regarding the mechanisms of actions of acyl-ghrelin is hypothalamic neurons [64, 65]. In these cells, acyl-ghrelin relies on 5' adenosine monophosphate-activated protein kinase (AMPK) [66–68]. Acyl-ghrelin AMPK-dependent signaling acts by two distinct mechanisms in hypothalamic neurons, in which intracellular calcium influx [69] and cytoplasmic nutrient sensors, such as AMPK [70] and mammalian target of rapamycin (mTOR), are the main targets triggered by the binding of acyl-ghrelin to GHS-R1a [71, 72].

AMPK activation leads to an inhibition of acetyl-CoA carboxylase (ACC) through posttranslational modifications [66]. The cellular outcome of this inhibition is increased mitochondrial metabolism due to consecutive activation of carnitine palmitoyltransferase 1 (CPT1) [73, 74]. Increased

fatty acid oxidation leads to the generation of reactive oxygen species (ROS) and consequently stimulates uncoupling protein 2 (UCP2) [75]. These events induce the expression of orexigenic neuropeptides and consequently feeding behavior [75]. AMPK activation through GHS-R1a can also be mediated by calcium calmodulin-dependent protein kinase-kinase 2 (CAMKK2) in response to elevated intracellular calcium concentrations [76–78]. There is also evidence for a dependency on Sirtuin 1 and p53 during AMPK activation through GHS-R1a signaling [79].

The cytoplasmic nutrient sensor mTOR has a key function in hypothalamic energy homeostasis [80]. Several reports indicate that the effects of acyl-ghrelin are mediated by mTOR signaling pathway activation [72, 81, 82]. Activation of this machinery is responsible for the phosphorylation of several transcriptional factors, which are key elements in the orexigenic response, such as forkhead box protein O1 (FOXO-1) and cAMP response element-binding protein (CREB) [71, 83]. These data indicate that acyl-ghrelin signaling pathway in hypothalamic neurons is dependent on the signaling machinery of nutrient sensing.

In immune cells, the mechanisms of acyl-ghrelin signaling are poorly explored. Avallone and colleagues [84] show that ghrelin signaling in macrophages is dependent on AMPK activation and peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) [84]. Both proteins have established anti-inflammatory roles [81–86]. Further studies are required to fully characterize acyl-ghrelin signaling in immune cells. The dependence of AMPK and PPAR $\gamma$  for the immunoregulatory features of acyl-ghrelin is consistent with the current understanding of ghrelin signaling events in hypothalamic neurons as well as the cellular modifications that immune cells undergo during the induction of an anti-inflammatory phenotype [85–87].

**3.1. Mechanisms Independent of Nutrient Sensors.** There are two physiological outcomes as resultant of ghrelin receptor activation that does not depend on the nutrient sensing machinery: (i) the activation of hypothalamic neurons that evoke feeding behavior [69] and (ii) growth hormone (GH) secretion by pituitary cells [88]. Both outcomes are a direct result of elevated calcium levels, with distinct mechanisms of action. In the first case, activation of GHS-R1a leads to a subsequent elevation of cyclic adenosine monophosphate (cAMP), mediated by the adenylate cyclase (AC)-protein kinase A (PKA) signaling pathway [89]. As a result, hypothalamic neurons involved in feeding behavior are activated [69]. In pituitary cells, activated GHS-R1a induces calcium release from endoplasmic reticulum, which results in the activation of phospholipase C (PLC)-inositol triphosphate (IP $_3$ )-protein kinase C (PKC) pathway [39, 88].

#### 4. Ghrelin, Obesity, and Inflammation

Tissue-secreted factors may disturb tissue homeostasis, which affects cellular and tissue metabolism and leads to systemic alterations [26]. Ghrelin and other factors regulate several aspects of metabolism and inflammation, which

result in improved or worsened insulin resistance and metabolic syndrome [90–92]. Obesity-mediated metabolic disturbances increase levels of several cytokines and chemokines [91, 93]. This generates a proinflammatory status, which is a potential risk factor for the development of inflammation-induced insulin resistance [94]. One of these secreted factors is the monocyte chemotactic protein-1 (MCP-1), which is induced by the NF $\kappa$ B pathway to recruit monocytes [95]. Because activation of GHS-R reduces NF $\kappa$ B activation in endothelial cells [96], ghrelin treatment could limit immune cell activation through inhibition of NF $\kappa$ B activation and subsequent MCP-1 secretion. This approach may lead to the development of new therapeutic approaches to treat T2D.

Metabolic imbalance induced by obesity leads to alterations in ATM population profile [97]. The M1 ATM macrophage population expresses the cell surface marker CD11c and secretes proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1) [98]. M1 macrophages also express high levels of iNOS (inducible nitric oxide synthase) [98, 99]. M2 macrophages express cell surface markers CD206, CD301, and CD163, secrete anti-inflammatory cytokines, such as interleukin-10 (IL-10), and express high levels of arginase-1 [15, 99]. M2 ATMs are often involved in homeostasis maintenance and tissue repair [15, 100]. The increase in the number of M1 ATM population in obesity is commonly accompanied with a reduction in M2 ATM population. This imbalance, with the predominance of a proinflammatory profile, inhibits the insulin-signaling pathway [94, 101, 102]. Thus, ATM function has an important role on metabolic syndrome and T2D development, which is frequently observed during obesity [99].

In obesogenic conditions, ghrelin levels are decreased [23] and levels of proinflammatory cytokines and adipokines, such as leptin, and liver-derived proteins, such as retinol binding 4 (RBP4), are increased [5, 103, 104]. Leptin is a proinflammatory adipokine, which inhibits ghrelin secretion [105] and worsens adipose tissue inflammation [5]. These data support the hypothesis that counter regulatory functions between leptin and ghrelin are an essential step for the maintenance of homeostasis CNS (food intake and energy expenditure regulation) and in immune responses [36].

#### 5. Immunoregulatory Functions of Ghrelin

The wide distribution of functional ghrelin receptors (GHS-R) and their expression in various immune cell populations have attracted the attention of the scientific community. Changes in ghrelin levels can directly affect immune responses and tissue homeostasis [36, 37]. Leukocytes, such as adipose tissue macrophages (ATMs), express GHS-R [106] and detect changes in energy status [107, 108]. Thus, ghrelin actions on ATMs may play a role in the maintenance of the tissue homeostasis, suggesting a link between the immune system and systemic metabolism in response to different physiological and pathological conditions such as obesity and insulin resistance [109, 110].

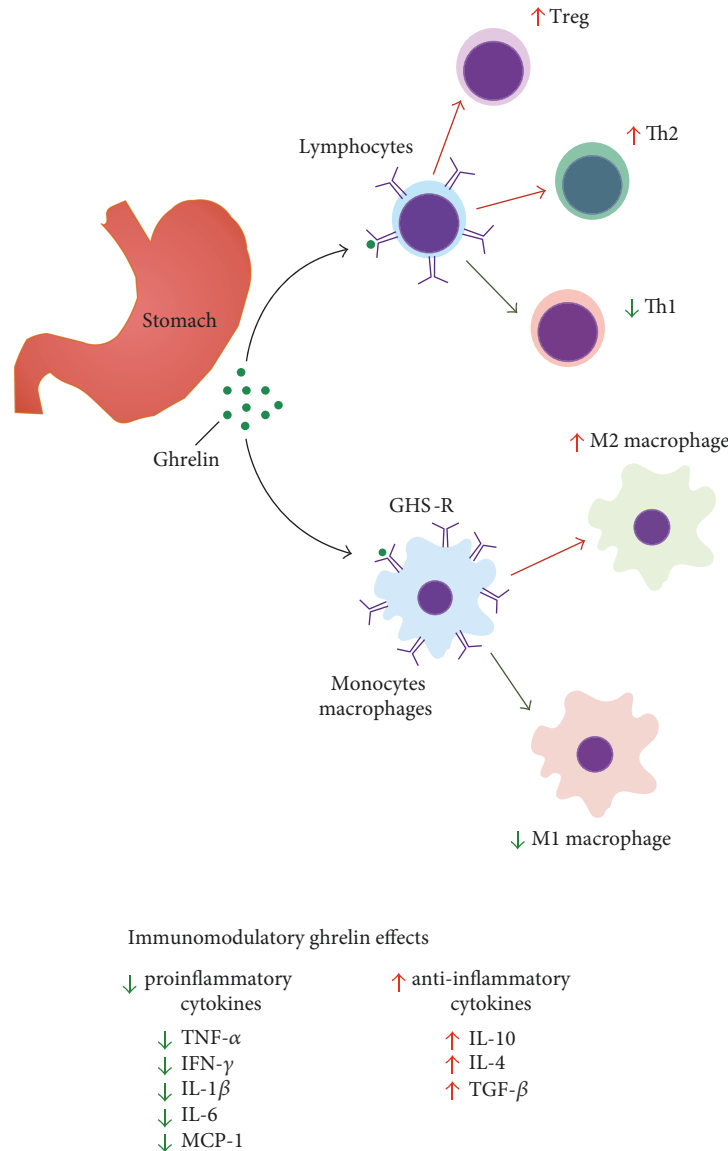


FIGURE 1: The immune roles of ghrelin. Ghrelin is secreted by X/A-like enteroendocrine cells. In the innate immune system, ghrelin acts on macrophages and induces an anti-inflammatory state (M2 profile) and inhibits proinflammatory macrophages (M1 profile). In the adaptive immune system, ghrelin exhibits an anti-inflammatory role. Ghrelin inhibits Th1 cells and increases the polarization of Th2 and regulatory T cells. These actions contribute to the reduced levels of proinflammatory cytokines and increased levels of anti-inflammatory cytokines.

Several studies support an immunoregulatory role for ghrelin [36, 37, 107, 111, 112]. Ghrelin acts on a diverse portfolio of leukocytes and directly alters immune cell function (Figure 1) [36, 37, 111, 112]. Dixit and colleagues [111] showed that ghrelin treatment *in vitro* inhibited the secretion of proinflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) in human monocytes, T cells, and peripheral blood mononuclear cells (PBMCs) [111]. Likewise, ghrelin treatment in human umbilical vein endothelial cells (HUVEC) reduced the secretion of IL-8 and MCP-1 and the activation of NF $\kappa$ B in response to TNF- $\alpha$  stimuli [96]. Also, rats submitted to endotoxic shock with lipopolysaccharide (LPS) and treated with ghrelin had higher rates of survival compared to controls [113]. This protective effect was mediated by ghrelin-specific

GHS-R receptor binding and resulted in reduced serum levels of TNF- $\alpha$ , IL-6, IL-8, and MCP-1 [96, 111]. Moreover, data suggests that mitogen-activated protein kinase phosphatase-1 (MKP-1) mediates the protective effect of ghrelin against endotoxic shock [114]. MKP-1 levels are reduced in inflammatory conditions, such as norepinephrine-induced sepsis, and lead to secretion of TNF- $\alpha$  [114]. Jacob and colleagues [114] reported that ghrelin treatment in septic rats increased gene and protein expression of MKP-1 [114]. This restoration of MKP-1 expression may partially explain the reduction in proinflammatory cytokines in response to ghrelin treatment. These data indicate that ghrelin can limit inflammation and plays an important role in metabolic and nonmetabolic inflammatory conditions.

*In vivo*, ghrelin has an anti-inflammatory and antinociceptive role [13, 34, 37, 111, 115–118]. Intraperitoneal administration of ghrelin in rats submitted to pain resulted in increased levels of serum IL-10 and TGF- $\beta$  and reduced pain score [118]. The anti-inflammatory action of ghrelin was also observed in a colitis model. Ghrelin treatment reduced the expression of TNF- $\alpha$ , INF- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, IL-15, IL-17, and IL-18 and increased IL-10 levels in colonic mucosa, which improved colitis score and survival rate in mice [37].

The anti-inflammatory roles of ghrelin can be extended to other inflammatory conditions, such as rheumatoid arthritis. Administration of the ghrelin agonist growth hormone-releasing peptide-2 (GHRP-2) reduced serum IL-6 levels and improved inflammation in arthritic rats [119]. Similar observations were obtained when peritoneal macrophages were treated with GHRP-2 *in vitro* [119]. Together, this data indicates that GHS-R can be used as a novel target for the treatment of acute and chronic inflammatory diseases.

Neutrophils play a fundamental role in immune response against pathogens and are regulated by ghrelin treatment. *In vivo* studies show that ghrelin treatment reduced neutrophil count in peritoneal lavage [63]. This was not attributed to enhanced apoptosis [120]. Neutrophils treated with ghrelin had increased phagocytic capacity and enhanced bactericidal capacity [120]. On the other hand, ghrelin treatment reduced neutrophil recruitment in the airways of subjects with chronic respiratory infections, which displayed reduced IL-8 and TNF- $\alpha$  levels in the sputum and improved overall inflammatory status [121].

Orlova and colleagues showed that ghrelin may affect dendritic cell- (DC-) mediated antigen presentation capacity. DCs treated with ghrelin had reduced capacity to induce the secretion of IL-17 and INF- $\gamma$  and enhanced capacity to induce secretion of IL-10 and TGF- $\beta$  from cocultured T cells [122]. Ghrelin also modulates thymic DCs. Ghrelin administration in the thymus induced the proliferation of DCs in aged mice [123], which contributes to a more effective maturation and response of effector and regulatory (Treg) T cell differentiation [124]. Ghrelin also regulates immune cell migration and proliferation. Ghrelin treatment reduced immune cell recruitment after LPS stimulation and induced the proliferation of Treg cells [34, 125]. Together, these data indicate that ghrelin has important effects in modulating T cells, especially regulatory T cells.

The role of adaptive immunity in the CNS is a growing topic of study. One of the most established models of the role of lymphocytes in CNS homeostasis is experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis [126]. This condition has a marked proinflammatory feature, mediated by T CD4<sup>+</sup> cells (Th1 and/or Th17 response) [125]. Ghrelin exerts therapeutic effects in EAE through the impairment of encephalitogenic Th1 and Th17 cells, and short-term ghrelin treatment reduces the clinical score of the disease [125]. This was associated with fewer infiltrated cells in the CNS and subsequent reduction in CNS inflammation. These effects were further improved by

the induction of regulatory T cells in mice [125]. Souza-Moreira and colleagues [125] showed that ghrelin treatment suppressed M1 phenotype in microglia and reduced T cell infiltration, which was consistent with findings previously described by Theil and colleagues [125, 127]. The immunomodulatory roles of ghrelin in CNS are a result of decreased expression of proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, which indicate a noteworthy anti-inflammatory property.

## 6. Contrasting Roles of Ghrelin in the Immune System

There is evidence supporting the immunoregulatory role of acyl-ghrelin [107] and its beneficial effects to treat chronic inflammatory syndromes, especially acyl-ghrelin immunoprotective properties during endotoxic shock [36]. However, in sepsis, ghrelin may contribute to the higher mortality seen in septic mice [63]. This higher mortality was attributed to reduced neutrophil and natural killer cell activity, which led to increased bacterial burden [63]. Nevertheless, other authors demonstrated beneficial effects of acyl-ghrelin in the same experimental model and attributed these effects to lower inflammation in the hippocampus, observed by the reduction of TNF- $\alpha$  and IL-6 levels in septic brains [112].

Desacyl-ghrelin is a non-GHS-R1a ligand and was previously described as the nonactive form of ghrelin (although some authors report that desacyl-ghrelin binds to GHS-R1a when supraphysiological concentrations are induced) [34, 39, 44, 128]. Recent reports suggest that desacyl-ghrelin alters macrophage polarization *in vitro* [129]. Desacyl-ghrelin treatment decreased expression of TNF- $\alpha$  and CD11c and increased expression of CD206 in the mouse macrophage cell line (RAW264.7) [129]. Similar results were observed by the treatment of RAW cells with acyl-ghrelin [130]. This suggests that ghrelin treatment reduced M1 proinflammatory macrophage and increased M2 macrophage polarization [129]. These results are compatible with the observation that in humans, monocytes are the main targets for the anti-inflammatory actions of acyl-ghrelin [111]. However, the effects of acyl-ghrelin or desacyl-ghrelin treatment in bone marrow-derived macrophages or ATMs remain to be established.

Knockdown of GHS-R in mice reduced expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and MCP-1 in WAT of mice fed with a high-fructose corn syrup, which was associated with improved insulin sensitivity and obesity in aged mice. Also, ablation of the GHS-R promoted a shift towards a M2 profile in ATMs [106]. GHS-R ablation also limited the proinflammatory phenotype of peritoneal macrophages and ATMs, which was observed by decreased expression of proinflammatory cytokines [99].

Although a variety of studies indicates that ghrelin has an anti-inflammatory role, the controversial actions of this peptide support the need to better understand the mechanisms by which ghrelin acts on immune cells in response to different immunological challenges.

## 7. Ghrelin at the Neuroimmune Interface

**7.1. Neuroprotective Effects of Ghrelin.** In extrahypothalamic areas, acyl-ghrelin has a strong anti-inflammatory role [131–134]. Microglial cells express GHS-R, and acyl-ghrelin administration downregulates proinflammatory cytokine expression through impairment of microglial cell expansion [131–133]. The neuroprotective effects of acyl-ghrelin were extensively studied by many research groups [135–137]. In Parkinson's disease, there is a progressive neuronal degeneration of dopaminergic neurons localized in the *substantia nigra* and a concomitant increase in microglial activation. GHS-R1a is widely expressed in dopaminergic neurons in the *substantia nigra* [136, 137]. It is proposed that the neuroprotective effects of acyl-ghrelin in a Parkinson's disease context are partially due to the induction of tyrosine hydroxylase expression in dopaminergic neurons, a pivotal enzyme in dopamine biosynthesis [135]. The administration of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a mitochondrial toxin, is a well-established model for Parkinson's disease studies, since there is a selective effect on dopaminergic neurons [138]. Mice treated with intraperitoneal acyl-ghrelin have lower levels of neuronal death and reduced dopamine loss [132, 133, 135]. Consistent with this phenotype, ghrelin treatment reduces proinflammatory markers, such as TNF- $\alpha$  and IL-1 $\beta$  [133]. It is proposed that the neuroprotective effects of ghrelin are also mediated through UCP2 activity [75, 135], since striatal neurons are dependent on UCP2 for optimal function [139–142].

Learning and memory retention also are influenced by acyl-ghrelin [143]. Intracerebroventricular [144] and intrahippocampal [145] acyl-ghrelin injections improve memory retention in rodents, which suggests a role for ghrelin in the molecular process of memory acquisition and/or consolidation. These results are reinforced by data published by Diano and colleagues [146], where spine density in the hippocampus is reduced in ghrelin knockout mice [146]. Data from Carlini and colleagues [144, 147] suggest that these beneficial effects are partially due to serotonergic inputs from dorsal raphe nucleus to the hippocampal circuits [147].

Several studies indicate that the brain is not a postmitotic structure in adult life [148–151]. Adult neurogenesis may be a potential therapeutic target for many neurodegenerative conditions [152–154]. The most explored structure in this context is the hippocampus [155]. There is evidence that acyl-ghrelin induces neurogenesis in brain structures related with cognition, such as the dentate gyrus of the hippocampus [156]. The classical target of ghrelin resides in the neuronal populations of the hypothalamus, and there are no reports to date describing the induction of neurogenesis by ghrelin. Therefore, neuroprotective effects of ghrelin in cognition-related structures may contribute to both the regulation of neurogenic events and the maintenance of mature resident cells [157–159].

The neuroprotective effects of ghrelin are also related to ischemic lesions, both *in vivo* and *in vitro* [160–162]. In these situations, there is insufficient blood flow into the brain. Treatment with acyl-ghrelin reduces ischemic lesions in mice

by mechanisms both dependent and independent of GHS-R1a [160–162]. Here, neuroprotection is determined as reduced infarct tissue and cell death [161, 163].

**7.2. Ghrelin, Stress, and Neuroinflammation.** In view with the increased incidence of psychological disturbances and obesity, many groups have investigated the dynamic contribution of obesity to the development of affective disorders and how affective disorders affect obesity. Hormones that regulate energy homeostasis, such as ghrelin, may play a role in mediating psychological disturbances [164].

Serum levels of acyl- and desacyl-ghrelin, preproghrelin, and GH are increased in rodents submitted to acute and chronic stress models [164–168]. The hypothesis that increased ghrelin levels could be due to a stress response is supported by the involvement of ghrelin in neuroprotection, memory, and motivation [143]. GHS-R knockout mice have depressive behavior, which is marked by social isolation [165]. Cummings and colleagues showed that the increased ghrelin levels lead to decreased depressive behavior in rodents submitted to forced swimming test [165]. Psychological stress appears to induce inflammatory responses and is associated with compartmental alterations characterized by depressive symptoms [169]. Elevated levels of proinflammatory cytokines are found in patients with depression [170]. IL-1 $\beta$  and TNF- $\alpha$  increase serotonin uptake and metabolism, which contribute to depressive behavior. These data suggest a link between inflammatory responses and compartmental diseases [170]. GHS-R expression in the basolateral complex of the amygdala, an important region for emotional processing in rodents and humans, strongly supports the involvement of ghrelin in the modulation of emotional status and memory [171, 172]. Thus, ghrelin treatment could be an effective approach against emotional disorders due to ghrelin anti-inflammatory properties [143, 170].

Alterations in endogenous ghrelin levels and action could lead to the development of psychiatric disturbances associated to stress [172, 173]; a better understanding of how ghrelin regulates emotional behavioral disturbances is needed. These studies may contribute to the development of new targets for the treatment of diseases associated with stress and inflammation.

**7.3. Ghrelin and Mediobasal Hypothalamus.** The hypothalamus is a CNS structure primarily involved in global metabolic regulation [35]. There are multiple hypothalamic nuclei involved with metabolic regulation, such as the arcuate nucleus (Arc), lateral hypothalamic area (LHA), and paraventricular nucleus (PVN) [35]. The current model stipulates that Arc neuronal populations work in a binary-like system. The anorexigenic response is mediated by proopiomelanocortin (POMC) neurons, and the orexigenic response is mediated by agouti-related protein (AgRP) expressing neurons [35]. The signals induced by key metabolic hormones and nutrients are perpetuated by other neuronal populations in different hypothalamic nuclei that are synaptically connected to AgRP/POMC neurons [35]. Ghrelin exerts its orexigenic actions through AgRP neurons exclusively, since POMC neurons do not express GHS-R [174].



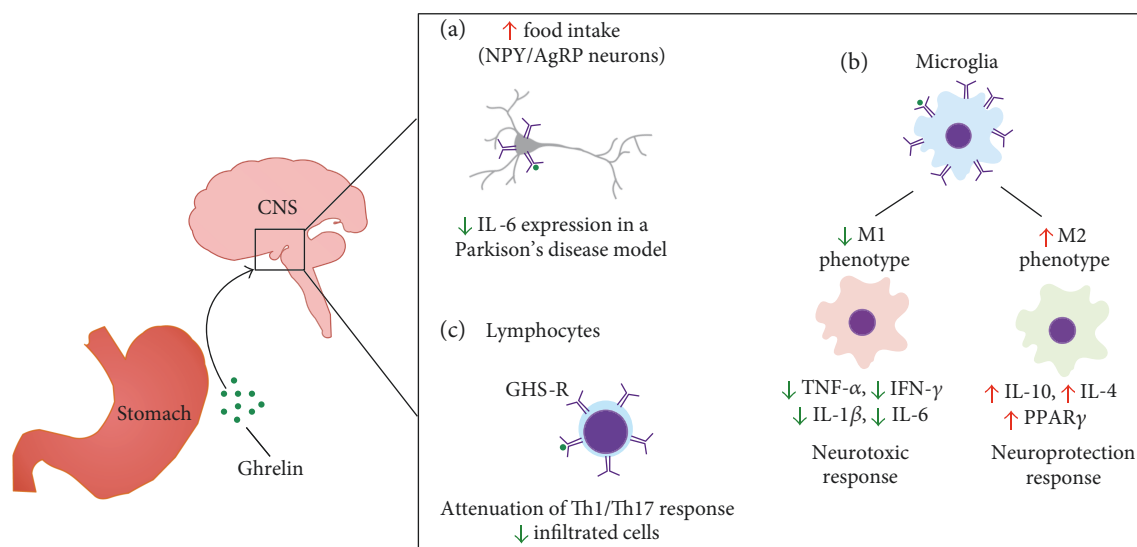


FIGURE 2: Ghrelin at the neuroimmune interface. Ghrelin has several functions in the CNS. (a) The classical orexigenic effect of ghrelin is mediated through activation of AgRP/NPY neurons in the mediobasal hypothalamus; it can also exert neuroprotective effects by diminishing IL-6 expression in striatal neurons in a Parkinson's disease model. (b) In microglia, ghrelin suppresses the proinflammatory phenotype and activates an anti-inflammatory program, which reinforces the neuroprotective role of ghrelin. (c) Infiltrated lymphoid cells are also decreased upon ghrelin treatment, with a marked reduction in Th1/Th17 responses.

It is known that consumption of high-fat diet (HFD) leads to activation of proinflammatory processes in the hypothalamus with marked deregulation of feeding behavior and defective energy expenditure responses [175–177]. This inflammatory process is activated in the early stages of HFD feeding, with increased cytokine expression within 24 hours after HFD intake [176]. The key cell type that coordinates inflammatory responses in CNS is the microglia [178]. Several reports in the past decade described the importance of microglial cells in the initiation and perpetuation of hypothalamic inflammation and consequently its repercussions [175–177]. Following an obesogenic stimulus, microglial cells are activated in a TLR4-dependent manner and rapidly expand, creating a proinflammatory environment. These actions are restricted to the hypothalamus in the early stages of “metabolic inflammation” [175]. A recent report demonstrated that inhibition of microglial expansion in the Arc partially restored the metabolic impairments due to an obesogenic environment [177]. Thus, microglial activation is one of the most important targets for the development of focusing on the CNS under obese conditions.

The activation of TLR4-dependent responses in resident Arc microglial cells leads to a defective ghrelin orexigenic effect in animals fed with a chow diet [179]. This data indicates that microglia can directly modulate energy homeostasis by affecting neighboring cell functions and might explain the counterintuitive phenomenon of ghrelin resistance in obesity and metabolic diseases. However, ghrelin resistance is a complex subject of study and cannot be reduced to isolated effects on microglial or neuronal cells in the hypothalamus [180]. For instance, it is not known whether ghrelin resistance is due to (i) reduced ghrelin receptor expression/translocation in the target cells, (ii) defective ghrelin transport through blood-brain barrier as observed with leptin

[181], or (iii) disrupted cellular homeostasis on ghrelin responsive cells, which is a similar process that leptin responsive cells undergo in obesogenic conditions [181, 182]. It is also possible that the physiological outcome is a combination of all of these factors. Another point to be clarified is whether neurons and glial cells are both resistant to the ghrelin signal and if there is temporal concordance in the establishment of ghrelin resistance in different cell types.

#### 7.4. Obesity and Immunity in the Central Nervous System.

The participation of Th1 or Th17 cells on hypothalamic impairment in metabolic diseases has yet to be clarified. It is known that T cells can penetrate the blood-brain barrier and act locally on the brain [183]. Thus far, there is no characterization of the participation of these cell types in the induction of metabolic inflammation on the hypothalamus. It is possible that ghrelin exerts an indirect immunomodulatory effect on adaptive immunity. As briefly discussed, Arc microglial activation can lead to an increase in local TNF [176]. This signaling has multiple effects and is one modulator of adaptive immunity in the periphery, which triggers adipose tissue lipolysis and raises triglyceride levels in the blood [184]. This in turn elevates the number of B cells and T cells with a Th1-type profile [184]. Thus, in obesity and obesity-related conditions, the immunomodulatory mechanisms of ghrelin have multiple points of actions and might not be restricted to the neural tissue (Figure 2).

Long-term studies indicate that bariatric surgery promotes a significant and sustained weight loss and recovery of metabolic parameters [185]. Several works have reported alterations in ghrelin levels during the postoperative period in patients submitted to different surgical strategies, which could be a result of altered body weight and feeding behavior [165, 186–206].

Faraj and colleagues [199] reported that ghrelin levels are dynamically modulated with weight loss [199] and patients with no changes in body weight do not have altered ghrelin levels. However, the role of ghrelin in postbariatric surgery is controversial [165, 186–206]. Besides, another relevant aspect that should be considered is the different methodological approaches used for the measurement of ghrelin, which could contribute to the discrepancy in ghrelin levels reported [207, 208].

Although the exact mechanisms by which bariatric surgery leads to reduced body weight are not completely understood, alterations in nutrient influx accompanied by increased ghrelin levels could lead to reduced proinflammatory marker expression, which will result in improved metabolic inflammation and the subsequent glucose homeostasis [199, 209–212].

## 8. Concluding Remarks

Ghrelin is not only a gastric peptide with CNS actions but it is also an important hormone/cytokine with important pleiotropic functions. The wide distribution of GHS-R1a in different cell types, including immune cells, indicates that ghrelin acts as a potent immunomodulator with powerful anti-inflammatory roles. The anti-inflammatory effects of ghrelin are observed in immune cells of both myeloid and lymphoid lineages. In macrophages/microglia, these anti-inflammatory properties are translated into increased secretion of anti-inflammatory cytokines, elevated M2/M1 ratio, and reduced proinflammatory cytokine expression. In lymphoid cells, ghrelin signaling leads to increased Th2 and Treg cell function. Therefore, ghrelin is a secreted hormone/cytokine with important anti-inflammatory roles in metabolically relevant organs, such as WAT and the hypothalamus. Ghrelin is a promising therapeutic strategy for the treatment of chronic inflammatory conditions, such as obesity. The therapeutic function of ghrelin is currently limited by its potent orexigenic properties. The paradoxical outcomes of ghrelin used to treat metabolic diseases are the induction of appetite and the anti-inflammatory roles. Thus, more studies are required to elucidate the molecular mechanisms of ghrelin actions as well as its application as a GHS-R agonist to treat obesity and insulin resistance in individuals.

## Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contributions

Jéssica Aparecida da Silva Pereira and Felipe Corrêa da Silva contributed equally to this work.

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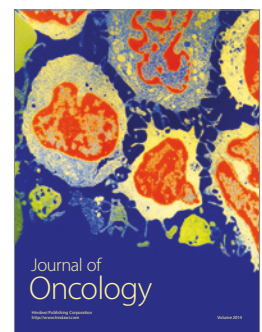
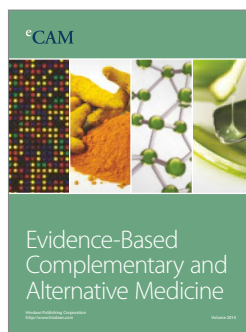
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## **APENDICE 2**

MINI-REVIEW

## Mitoimmunity—when mitochondria dictates macrophage function

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### Abstract

In the past decade, several reports have appointed the importance of mitochondria in the immune response. Our understanding of mitochondria evolved from a simple supplier of energy into a platform necessary for immunoregulation. Proinflammatory responses are associated with enhanced glycolytic activity and breakdown of the TCA cycle. Mitochondrial reactive species of oxygen (mROS) are key regulators of classically activated macrophages, with substantial impact in the anti-microbicidal activity and pro-inflammatory cytokine secretion of macrophages. The inflammasome activation in macrophages is dependent on mROS production and mitochondrial regulation and mitochondrial dynamics and functionality direct impact inflammatory responses. Alternative activated macrophage metabolism relies on fatty acid oxidation, and the mechanism responsible for this phenotype is not fully elucidated. Thus, cellular metabolism and mitochondria function is a key immunoregulatory feature of macrophage biology. In this review, we will provide insights into recently reported evidences of mitochondria-related metabolic nodes, which are important for macrophage physiology.

**Keywords:** inflammation; macrophage; metabolism; mitochondria

### Introduction

Mitochondria are organelles present in a broad spectrum of eukaryotes and have the primary function of providing energy for cellular processes. Mitochondria appeared from the engulfment of proteobacterium by a eukaryotic progenitor (Lane and Martin, 2010). Mitochondria are morphologically composed of two functionally distinct membranes—the permeable/outer and impermeable/inner membrane (Sazanov, 2015). The energetic pathways in mitochondria are complex processes, which use electro-chemistry energy from electrons to produce the energy carrier molecule adenosine triphosphate (ATP). These processes happen in the mitochondrial matrix and involve the protein complex called Electron Transport Chain (ETC). The global coordination of energy through mitochondria is highly dynamic and mostly dependent on the nutritional status (Cantó et al., 2010; Hardie, 2011; Mihaylova and Shaw, 2011). Mitochondrial biogenesis and function is directly regulated by the availability of

mitochondria-related molecules, such as ATP and nicotinamide adenosine nucleotide (NADH) (Cantó et al., 2010; Hardie, 2011; Mihaylova and Shaw, 2011).

Recently, several reports demonstrated the importance of mitochondria in immunity (Bae et al., 2012; Van den Bossche et al., 2012, 2016 Tannahill et al., 2013; Palsson-McDermott et al., 2015; Millet et al., 2016; Nemeth et al., 2016). Immune cells, such as macrophages, once activated mount an immune response that involves the production of cytokines, lipids, reactive species of oxygen (ROS), and cellular rearrangements (O'Neill, 2015). To appropriately respond, immune cells are extremely dependent on mitochondrial function, putting this organelle as the central platform for immune regulation (Mills et al., 2017). Mitochondria-derived factors are able to regulate inflammatory events, such as the activation of innate immunity receptors and the subsequent cytokine and ROS production (Pastorino and Hoek, 2008; Zhang et al., 2010; Horner et al., 2011; Nakahira et al., 2011; Shimada et al., 2012; Moon et al., 2015; Weinberg et al., 2015; West et al., 2015; Wolf et al., 2016).

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### Metabolic regulation of macrophage polarization

Macrophages spans a spectrum of phenotypes and the optimal constellation of markers to define pro- and anti-inflammatory macrophages is still evolving. Nevertheless, in a simplified manner, macrophages can assume two distinct profiles, called M1 or M2, which are dependent on the stimuli employed during its activation (Castoldi *et al.*, 2015; Weinberg *et al.*, 2015). M1 or classically activated macrophages respond to bacterial lipopolysaccharide (LPS) and Th1-type of cytokines, such as IFN- $\gamma$  (Arango Duque and Descoteaux, 2014; Moraes-Vieira *et al.*, 2014; Castoldi *et al.*, 2015; Pereira, 2017). M1 macrophages are involved in immune responses against pathogens and are characterized by the secretion of pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$  (Arango Duque and Descoteaux, 2014; Moraes-Vieira *et al.*, 2014; Pereira, 2017). In contrast, M2 or alternatively activated macrophages participate in tissue repair and homeostasis, orchestrating the tissue immune responses (Colegio *et al.*, 2014). The polarization of M2 macrophages is induced by Th2-type of cytokines, such as IL-4, IL-5, and IL-13, and these macrophages produce preferentially anti-inflammatory cytokines, such as IL-10 (Colegio *et al.*, 2014).

The cascade of events that follows activation of macrophages leads to a metabolic profile switch, which will directly impact macrophages function (Mills *et al.*, 2017). Depending on microenvironment cues, there is a marked preference for glycolytic over oxidative metabolism, even in the presence of oxygen. This phenomenon is called “Warburg effect,” which is commonly found in proinflammatory and classically activated M1 macrophages (Weinberg *et al.*, 2015). The switch from oxidative metabolism into aerobic glycolysis is an important factor for macrophage polarization. Moreover, an accurate regulation of mitochondrial function during this process is a critical step for macrophage-induced cytokine secretion and for the successful elimination of pathogens (Weinberg *et al.*, 2015).

In general terms, pro-inflammatory cells exhibit a more glycolytic profile whereas anti-inflammatory cells rely mostly on oxidative metabolism (Bae *et al.*, 2012; Van den Bossche *et al.*, 2012, 2016; Tannahill *et al.*, 2013; Palsson-McDermott *et al.*, 2015; Millet *et al.*, 2016; Nemeth *et al.*, 2016). The disruption of mitochondrial metabolism and glycolysis enhancement in proinflammatory macrophages promotes the synthesis of metabolites which are necessary for the immune response and generates nicotinamide adenine dinucleotide phosphate (NADPH), which supports ROS production and fatty acid synthesis (O’Neill, 2015). Also, in classically activated macrophages there are structural modifications in the ECT, which allow mitochondrial ROS (mROS) generation (Garaude *et al.*, 2016; Mills *et al.*, 2016).

The alternative polarization of macrophages, known as the anti-inflammatory or repairing phenotype, is characterized

by a distinct mitochondrial-dependent metabolic profile (Vats *et al.*, 2006a; Huang *et al.*, 2014, 2016; Namgaladze and Brune, 2014; Van den Bossche *et al.*, 2015). In mitochondria, macromolecules are oxidized for energy generation. For example, in the cytoplasm, glucose is converted into pyruvate, which is oxidized inside the mitochondria through several reactions of the TCA (Ernster and Schatz, 1981). Furthermore, M2 macrophages also require oxidative metabolism, particularly fatty acid oxidation (Vats *et al.*, 2006b; Huang *et al.*, 2014, 2016; Namgaladze and Brune, 2014; Van den Bossche *et al.*, 2015).

### Inflammasomes at the immunometabolic interface

Inflammasomes are molecular complexes formed upon response to a variety of physiologic and pathogenic stimuli. Inflammasomes are key nodes in innate immunity with direct impact on pathogen and dysfunctional/dead cells clearance (Guo *et al.*, 2015). The final outcome of inflammasome activation is the secretion of IL-1 $\beta$  and IL-18, two important proinflammatory cytokines (Guo *et al.*, 2015). The two best-characterized inflammasomes are the NLRP3 and NLRC4 and their activation is highly dependent on mitochondrial function and alterations in the mitochondrial system directly affect the inflammasome activity (West *et al.*, 2011a; Tannahill *et al.*, 2013; Weinberg *et al.*, 2015; Mills *et al.*, 2016).

A report published by Tannahill *et al.* (2013) showed that LPS-activated macrophages have increased levels of succinate, which directly stabilize hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), resulting in the subsequent release of IL-1 $\beta$ . Supporting this data, Mills and colleagues showed that increased oxidation of succinate in mitochondria, via succinate dehydrogenase (SDH), combined with elevated mitochondrial membrane potential led to mROS production (Tannahill *et al.*, 2013). The same group demonstrated that increased levels of succinate are responsible for HIF-1 $\alpha$  stabilization and the subsequent IL-1 $\beta$  secretion (Weinberg *et al.*, 2015). This succinate/Hif-1 $\alpha$  axis is dependent on the activity of the enzyme prolyl hydroxylase (PHD) (Tannahill *et al.*, 2013; Mills *et al.*, 2016). Bone marrow derived macrophages (BMDMs) treated with  $\alpha$ -ketoglutarate, a PHD substrate, led to the inhibition of the succinate accumulation. This inhibition reduced expression and secretion of IL-1 $\beta$  in a HIF-1 $\alpha$ -dependent manner (Tannahill *et al.*, 2013). Lampropoulou *et al.* (2016) also explored the Hif-1 $\alpha$ /IL-1 $\beta$  axis and found that itaconate, an anti-bacterial component able to inhibit the isocitrate lyase in bacteria and found in abundance in the mitochondria of activated macrophages, inhibits SDH activity. This inhibition results in an anti-inflammatory profile (Lampropoulou *et al.*, 2016; Meiser *et al.*, 2016). The levels of itaconate modify mitochondrial respiratory fluctuations in LPS-activated macrophages

(Lampropoulou et al., 2016), which directly impacts pro-inflammatory cytokine production.

The activation of extracellular Toll-like receptors (TLR1, TLR2, and TLR4) induces mROS production in a mechanism that is dependent on the translocation of tumor necrosis factor receptor-associated 6 (TRAF6) into the outer membrane of mitochondria (West et al., 2011a). There, TRAF6 interacts with evolutionary conserved signaling intermediate in Toll pathways (ECSIT) (Kopp et al., 1999; West et al., 2011a). TRAF6 promotes ECSIT ubiquitination, which is required for induction of mROS production and the subsequent enhancement of macrophage anti-bactericidal activity (West et al., 2011a). These authors also demonstrated that these events are exclusive of extracellular TLRs (West et al., 2011b). mROS are not involved with antiviral responses, which are mediated by intracellular pattern recognition receptors (West et al., 2011b).

It is clear that the regulation of IL-1 $\beta$  secretion mediated by the inflammasome is dependent on mitochondrial function (Zhou et al., 2011; Iyer et al., 2013; Wynosky-Dolfi et al., 2014; Park et al., 2015; Zhong et al., 2016; Traba et al., 2017). Zhou et al. (2011) demonstrated that mROS activates the NLRP3 inflammasome machinery. Once activated, the NLRP3 inflammasome components co-localize with mitochondrial clusters (Zhou et al., 2011). This indicates that the NLRP3 inflammasome machinery is able to sense mitochondrial signals (Zhou et al., 2011). Mitochondria is essential for inflammasome regulation (Wynosky-Dolfi et al., 2014). For instance, aberrant mitochondrial dynamics leads to increased secretion of IL-1 $\beta$  by macrophages (Park et al., 2015).

Zhong et al. (2016) also explored the regulatory loop between key transcriptional effectors involved in immune cell activation with the NLRP3 inflammasome machinery. The NF $\kappa$ B is a classical proinflammatory transcriptional factor engaged with the expression of proinflammatory cytokines, chemokines, and several other components of the innate immunity (Zhong et al., 2016). The activation of inflammasome induces mitochondrial damage/dysfunction (Weinberg et al., 2015). The NF $\kappa$ B signaling adaptor p62 recognizes damaged mitochondria, and induces mitochondria autophagic clearance (Zhong et al., 2016). Ablation of p62 in macrophages induces excessive secretion of IL-1 $\beta$  and accumulation of damaged mitochondria (Zhong et al., 2016). Thus, NF $\kappa$ B signaling components are able to restrain excessive inflammation through inhibition of mitochondrial damage, which limits the NLRP3 inflammasome activation. Other groups also studied the importance of damaged mitochondria clearance in immune cells. The process of removing damaged or excessive mitochondria is called mitophagy, and is a type of selective autophagy critical for maintaining cellular homeostasis and mitochondrial population (Ding and Yin, 2012). Defects in mitophagy are associated with accumulation of damaged mitochondria and

mROS, which leads to pathophysiologic conditions. Kim et al. (2016) demonstrated that stress-inducible protein (SESN2) plays a role in promoting mitophagy in macrophages in response to NLRP3 inflammasome activation. *Sesn2*-deficient mice have defects in mitophagy that leads to hyperactivation of NLRP3 and increased mortality in animal models of sepsis (Kim et al., 2016). Recently, Eddie Ip and colleagues demonstrated a role for IL-10 in mitophagy. They showed that the absence of IL-10 results in the accumulation of damaged mitochondria in macrophages. This results in dysregulated activation of the NLRP3 inflammasome and the subsequent increased secretion of IL-1 $\beta$ , which contributes to the aggravation of colitis (Ip et al., 2017). Furthermore, Esteban-Martínez et al. (2017) also described a role for mitophagy in regulating the glycolytic shift associated with M1 macrophage polarization.

### Concluding remarks

Mitochondria are essential regulators of innate immunity. During inflammation there are several alterations in the microenvironment and cellular processes, which are sensed by the mitochondria. Understanding the dynamic relationship between mitochondria and immune cell physiology is essential to better comprehend the pathways involved in inflammation initiation and resolution. We still know little about the role of mitochondria during immune responses. We must better comprehend how this organelle modifies the outcome of immune cell activation or inhibition. Understanding the importance of mitochondria in the regulation of immune responses may lead to the discovery of unknown metabolic pathways, which may lead to the development of new treatments for inflammatory diseases.

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## **APENDICE 3**

## REVIEW

# Leptin in the regulation of the immunometabolism of adipose tissue-macrophages

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## Abstract

Obesity is a pandemic disease affecting around 15% of the global population. Obesity is a major risk factor for other conditions, such as type 2 diabetes and cardiovascular diseases. The adipose tissue is the main secretor of leptin, an adipokine responsible for the regulation of food intake and energy expenditure. Obese individuals become hyperleptinemic due to increased adipogenesis. Leptin acts through the leptin receptor and induces several immunometabolic changes in different cell types, including adipocytes and *Mφs*. Adipose tissue resident *Mφs* (ATMs) are the largest leukocyte population in the adipose tissue and these ATMs are in constant contact with the excessive leptin levels secreted in obese conditions. Leptin activates both the JAK2-STAT3 and the PI3K-AKT-mTOR pathways. The activation of these pathways leads to intracellular metabolic changes, with increased glucose uptake, upregulation of glycolytic enzymes, and disruption of mitochondrial function, as well as immunologic alterations, such as increased phagocytic activity and proinflammatory cytokines secretion. Here, we discuss the immunometabolic effects of leptin in *Mφs* and how hyperleptinemia can contribute to the low-grade systemic inflammation in obesity.

## KEYWORDS

obesity, inflammation, leptin, immunometabolism

## 1 | INTRODUCTION

Obesity is a chronic disease characterized by excessive lipid accumulation in the white adipose tissue (WAT) in response to the imbalance

between food intake and energy expenditure.<sup>1</sup> Currently, obesity is one of the most prevalent diseases in the world. The World Health Organization estimates that about 650 million adults are obese, showing body mass index (BMI) equal or over 30, which is frequently accompanied by other health disturbances, such as type 2 diabetes (T2D), cardiovascular diseases, and metabolic syndrome, and thus, impaired quality of life and life expectancy.<sup>2-6</sup>

The white adipose tissue is extremely important for nutrient storage, which confers to WAT an important role in the maintenance of energetic homeostasis.<sup>7-10</sup> Since WAT is constituted by a variety of immune and non-immune cells, the alterations in the visceral WAT promoted by obesity are characterized by increased lipid accumulation in white adipocytes and by the infiltration of pro-inflammatory immune cells in this tissue, which leads to the development of chronic low grade inflammation.<sup>11</sup>

Several works have reported that these alterations in visceral WAT are an important risk factor to the development of obesity-related disorders, such as T2D and insulin resistance.<sup>3,4,10,12,13</sup> The

Abbreviations: ACAT-1, Acetyl-CoA acetyltransferase; AgRP, agouti protein; AMPK, 5' AMP-activated protein kinase; ARG-1, arginase-1; ATM, adipose tissue resident *Mφ*; BMI, body mass index; CD, cluster of differentiation; CPT-1, carnitine palmitoyltransferase-1; Db, Diabetes (gene); DC, dendritic cell; DIO, diet-induced obese; DTH, delayed-type hypersensitivity; EIC, experimentally induced colitis; FAO, fatty acid oxidation; FFA, free fatty acid; FIP, fibro-inflammatory progenitors; GLUT1, glucose transporter 1; HFD, high fat diet; HIF-1 $\alpha$ , Hypoxia-inducible factor 1-alpha; ILC, innate lymphoid cell; iNKT, invariant natural killer cell; IRF4, interferon regulatory factor 4; IRS, insulin receptor substrate; JKN, JunN-terminal kinase; KC, Kupffer cell; LDH, lactate dehydrogenase; Lep-R, Leptin receptor; Mgl, *Mφ* galactose-type lectin; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; NPY, neuropeptide Y; Ob, Obese (gene); Ob-R, leptin receptor; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PGC-1 $\beta$ , PPARgamma-coactivator-1beta; PKM2, pyruvate kinase M2; POMC, proopiomelanocortin; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; PTP1B, phosphotyrosine phosphatase 1B; rAAV, recombinant adeno-associated virus; ROS, reactive oxygen species; SAT, subcutaneous adipose tissue; SIRT1, Sirtuin 1; SOCS3, cytokine signaling suppressor 3; SQ-WAT, subcutaneous adipose tissue; T2D, type 2 diabetes; UCP1, uncoupling protein 1; VAT, visceral adipose tissue; WAT, white adipose tissue.

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increased susceptibility to develop such disorders has a direct association with immune imbalance, which is marked by the excess of pro-inflammatory cytokines and reduction of anti-inflammatory cytokines promoted by the alterations in the polarization status of adipose tissue resident M $\phi$ s (ATMs).<sup>11,14,15</sup>

Besides the role of WAT for nutrient storage, this organ has an important participation in the regulation of a variety of metabolic processes.<sup>9</sup> WAT secretes several hormones/cytokines, which are named adipokines.<sup>16</sup> Adipokines can be differentially secreted in different portions of the adipose tissue.<sup>17</sup> Also, adipokines are involved in the adipocyte maturation, systemic nutritional communication, and metabolic regulation of a variety of cellular types.<sup>2,9,17</sup> In addition, some adipokines, such as leptin, are increased during obesity, which support their involvement in obesity-associated cardiovascular disease, insulin resistance, and immune imbalance.<sup>2,9,17</sup> This indicates that adipokines can be a link between the metabolism and the immune system.<sup>2,8,9,13</sup>

## 2 | LEPTIN AND LEPTIN RECEPTOR—A BRIEF HISTORY

The discovery of leptin is dated in the year of 1950, when the first observation of the recessive genetic alterations that happened in C57BL/6 animals, and led the animals to the development of morbid obesity was registered.<sup>18</sup> The gene located in chromosome 6, in which the mutation was observed, was named *Obese (ob)* due to the morbid obesity phenotype generated in response to its mutation. Accordingly, the animals in which this gene is mutated were given the nomenclature *ob/ob*.<sup>18</sup>

The cloning of the *ob* gene enabled the identification of a highly conserved protein with 167 amino acids in human and mice, which is secreted according to the amount of adipose tissue mass, and is present in high levels in the bloodstream of obese individuals and is absent in *ob/ob* animals.<sup>19–22</sup> The administration of this protein was reported to promote the reduction of food intake and weight gain in *ob/ob* mice and diet-induced obese animals (DIO), which was a key step in the development of several new studies aimed at understanding the mechanisms involved in regulating metabolism and the pathogenesis of obesity.<sup>21,23,24</sup>

Leptin, from greek *leptós*, which means thin, is a 16 kDa monomeric nonglycosylated protein secreted as a product of the *ob* gene.<sup>21</sup> Leptin is mainly secreted by WAT, and this adipokine belongs to the superfamily of cytokines due its elevated similarity with others cytokines, such as IL-6, IL-12, and G-CSF.<sup>25,26</sup> The elucidation of leptin crystal structure enabled the characterization of leptin as a pleiotropic protein, which can act as a link between immune responses and the neuroendocrine system.<sup>20,25,27,28</sup>

The identification of leptin receptor is dated about 20 years after the first report of leptin.<sup>29</sup> Similar as the leptin discovery, the leptin receptor was identified through studies involving animals with a spontaneous genetic mutation in the *Diabetes (db)* gene, which is located in chromosome 4.<sup>29,30</sup> The mutation in *db* gene generated a

phenotype characterized by hyperphagia, obesity, hyperglycemia, and hyperinsulinemia, and the animals in which this mutation was first observed were termed *db/db*.<sup>31,32</sup> The phenotypic similarity between the genetic mutation of *ob* and *db* gene took the scientific community interest to the necessity of more studies with *db/db* and *ob/ob* mice. In this way, parabiosis studies performed by Coleman<sup>31</sup> were a major breakthrough to leptin functional studies.<sup>31,33</sup> Coleman observed that one factor, later named leptin, was absent in *ob/ob* mice.<sup>31</sup> Also, the inability to respond to this factor could be the reason for the metabolic disturbances observed in *db/db* mice.<sup>20</sup>

Few years later, Bray and York<sup>30</sup> showed that hypothalamic damage leads to excessive food intake and weight gain alterations, which results in obesity. These studies were determinant for the hypothesis that leptin could act in the hypothalamus and thus, promote a signaling mechanism to induce the sensation of satiety.<sup>22,23,30,34</sup> However, the description of the leptin receptor, named Ob-R or Lep-R, happened just in 1995, after cloning and identification of a class I receptor that belongs to the superfamily of the gp130 cytokine receptors, which are widely distributed in a variety of cellular types, and are responsible for leptin actions.<sup>29,35–37</sup> Since then, several works were conducted to elucidate how leptin modulates the function of a variety of cells, its mechanism of secretion and its involvement in the regulation of immune responses.<sup>19,21–24,38</sup>

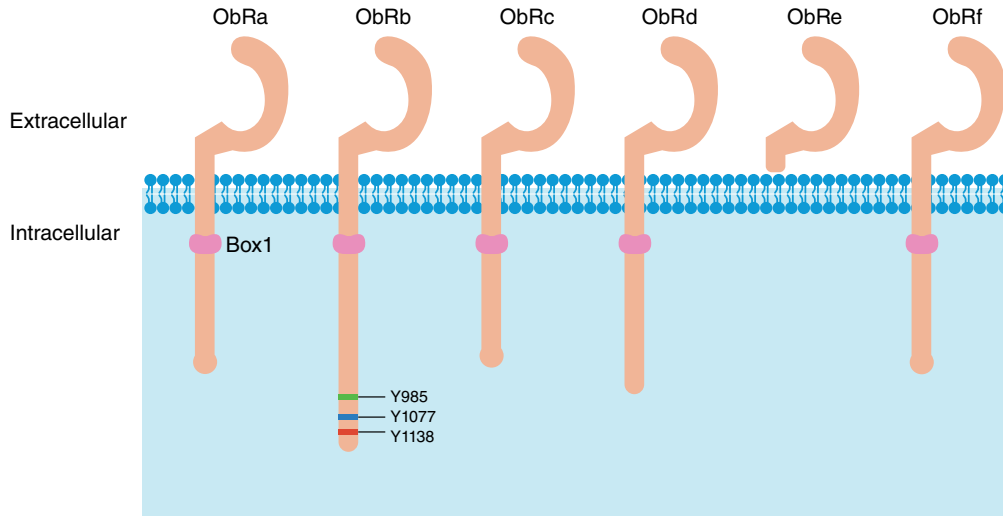
## 3 | LEPTIN RECEPTORS AND SIGNALING

Leptin receptors are widely distributed in a variety of cell types, and have a key role in the regulation of central and peripheral actions of leptin.<sup>29,35–37,39</sup> There are 6 isoforms of leptin receptor (Ob-R), which have high homology between humans and rodents.<sup>29,33,40</sup> These receptors are encoded by the *lepr* gene (humans) and *db* gene (mice), situated in the chromosome 4, and every isoform shows high affinity for leptin.<sup>41,42</sup>

The leptin receptor isoforms are distinguished by the letters a-f, which are classified in long (Ob-Rb), short (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Rf), or secreted (Ob-Re) forms.<sup>41,42</sup> In general, leptin receptors have an extracellular invariable domain, with 816 aas, and a variant intracellular domain, except the isoform Ob-Re, which does not have a transmembrane domain (Fig. 1).<sup>33,43,44</sup>

The multiple isoforms of the leptin receptor are generated in response to an alternative splicing, which results in the generation of proteins that have intracellular domains with different sequences and lengths,<sup>45–48</sup> ranging from 34 aas (short isoform) to 303 aas in its long isoform.<sup>33</sup> The intracellular domain of Ob-R receptors presents a conserved portion (box1), constituted by 6–17 aas, which have a key role in the association and activation of the tyrosine kinase JAK2, and another facultative domain (box2), which has 49–60 aas and is required for maximum activation of JAK2.<sup>43,49</sup>

The most well-characterized isoform of leptin receptor is the long form, Ob-Rb, which is present in the most varied cell types, such as M $\phi$ s,  $\beta$ -pancreatic cells, endothelial cells, among others.<sup>25,39</sup> It is mostly found in the hypothalamus,<sup>44</sup> in areas responsible for the



**FIGURE 1** Leptin receptor isoforms and signaling. There are 6 isoforms of leptin receptor (Ob-R) and they are classified as long (Ob-Rb), short (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Rf), and secreted (Ob-Re). Leptin receptors have an extracellular invariable domain, containing 816 amino acids, and intracellular domains that differ in sequences and lengths. The intracellular domain contains a conserved portion (box1), with a key role in the activation of JAK2, and a facultative domain (box2), required for maximum activation of JAK2. The long isoform (Ob-Rb) is present in several cell types, including M $\phi$ s. Ob-Rb presents 3 conserved tyrosines in their elongated intracellular domain required for activation of JAK2-STAT3 pathway. The short isoforms lack the sequences necessary for signal transduction and are involved in the transport and degradation of leptin in different tissues. The secreted isoform (Ob-Re) acts as a soluble transporter of leptin and promotes the association of leptin with hypothalamic Ob-Rb receptors

control of appetite and body weight.<sup>25,37</sup> Ob-Rb receptor is the only isoform that has an elongated intracellular domain with the required sequences for signal transduction. This isoform has 3 conserved tyrosines in its intracellular domain that allow the binding of molecules responsible for the activation of different signaling pathways, being the only isoform capable of promoting the activation of the JAK-STAT3 cellular signaling pathway.<sup>43,47,49,50</sup> Several studies have demonstrated that one of the main forms of leptin activity occurs through the activation of the pathway JAK2-STAT3, which has been pointed out as one of the main mechanisms by which leptin promotes satiety and control of energy expenditure.<sup>27,35,36,51,52</sup>

In response to the association of leptin, the Ob-Rb receptor undergoes a process of dimerization and conformational change, which induces the catalytic activity of the protein JAK2, and thus, leading to the phosphorylation of 3 tyrosine residues located in the Ob-Rb receptor, which promotes the activation of different signaling proteins.<sup>53-55</sup> The Tyr1138 tyrosine phosphorylation promotes the activation of STAT3, which after being phosphorylated by JAK2 dimerizes and translocates to the nucleus, where it induces the expression of anorexigenic peptides such as proopiomelanocortin (POMC), and the suppression of orexigenic peptides such as the neuropeptide Y (NPY) and agouti protein (AgRP). In addition, leptin binding to its receptor also regulates the expression of the cytokine signaling suppressor 3 (SOCS3), which is responsible for STAT3 inhibition, and the subsequent disruption of leptin-induced actions.<sup>27,35,51,53,54,56-58</sup>

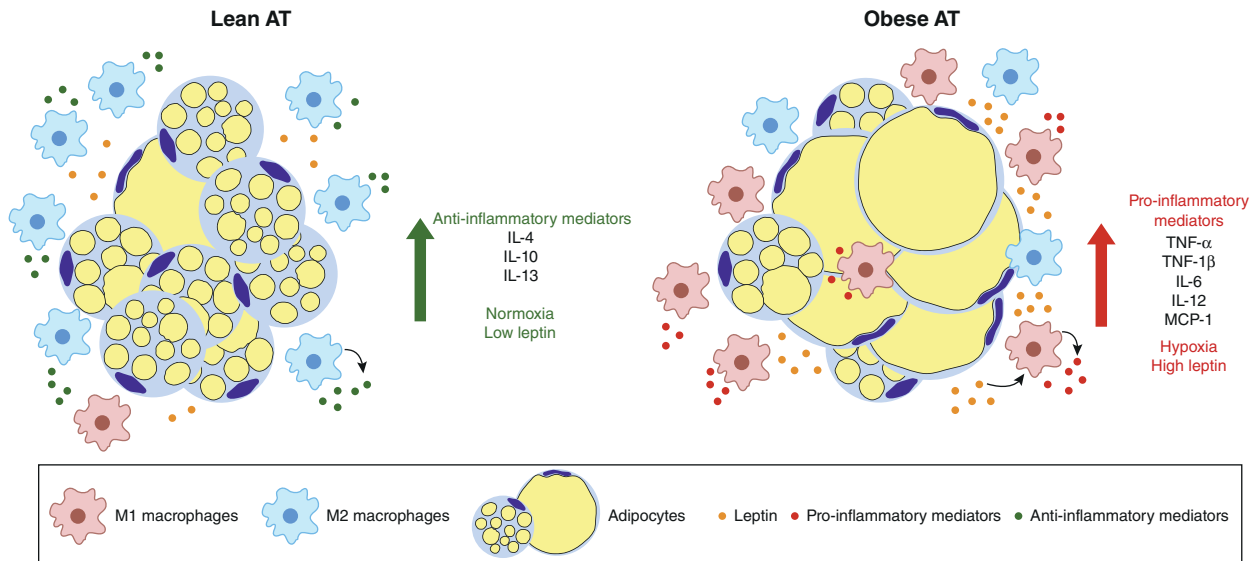
Other signaling pathways independent of STAT3 are regulated by leptin in different cell types.<sup>27,54,59-61</sup> Different groups reported the induction of pathways such as ERK, PI3K/Akt/mTOR, p38 MAPK, and AMPK/SIRT1 in response to leptin binding to

Ob-Rb receptors.<sup>25,27,35,59,62</sup> Some reports show that the activation of JAK2 promotes the phosphorylation of the insulin receptor substrate (IRS), which induces the activation of PI3K and consequently of protein kinase B, also known as Akt, thus promoting the modulation of anorexigenic/orexigenic peptide levels.<sup>63</sup> STAT3-independent mechanisms are also observed in relation to the control of JAK2 activation and termination of leptin signaling, in which phosphotyrosine phosphatase 1B (PTP1B), a protein present on the surface of the endoplasmic reticulum, is responsible for promoting dephosphorylation of JAK receptor after internalization of the Ob-Rb receptor.<sup>25,64,65</sup> The effects of leptin in the CNS have been extensively studied and described in other works.<sup>66-68</sup> We will not extend the discussion on this topic, since this is not the main focus of this review.

#### 4 | EFFECTS OF PERIPHERAL AND CENTRAL LEPTIN IN IMMUNE RESPONSE

Central leptin resistance affects hypothalamic nuclei and leads to a disruption in the regulation of food intake.<sup>69</sup> The white adipose tissue also develops leptin resistance, marked by a decrease in STAT3 activation in diet-induced obese mice, that leads to reduced ObRb expression.<sup>70,71</sup> However, efficient leptin signaling still happens in other tissues under obesogenic conditions.<sup>69</sup> Previous work from Enriori et al. showed that sympathetic outflow stimulated by leptin in brown adipocytes continues to work despite the central leptin resistance caused by obesity.<sup>72</sup>

Elevation of leptin concentrations in the cerebrospinal fluid and specific brain sites is responsible for leptin effects on food intake.<sup>73</sup>



**FIGURE 2** Leptin role in adipose tissue immunoregulation. Under physiological conditions, ATMs present anti-inflammatory characteristics, with secretion of IL-10, IL-4, and IL-13. Obesity leads to exacerbated adipocyte size and number, which results in increased leptin secretion that is accompanied by the inflammatory phenotype observed in obesity. Leptin induces the up-regulation of MCP-1 in the adipose tissue, consequently increasing the number of infiltrating proinflammatory blood monocyte-derived M $\phi$ s. Obese adipose tissue, with excessive leptin levels becomes hypoxic. Leptin also induces the expression of HIF-1 $\alpha$  in ATMs, favoring the production of proinflammatory mediators. Elevated leptin concentrations in obese adipose tissue promotes a shift in M $\phi$  immunometabolic profile, leading to higher secretion of proinflammatory cytokines, such as TNF- $\alpha$ , IL-6, IL-12, and IL-1 $\beta$

Yet, when administered by intravenous injection, leptin is not able to reduce food intake, due to the limitations of leptin transport once it comes across the blood-brain barrier.<sup>73</sup>

Inflammatory signals, such as LPS, stimulate leptin expression and release from the adipose tissue.<sup>74,75</sup> Peripheral administration of leptin reverts the enhanced thymocyte apoptosis in ob/ob mice and increases Th1 pattern of cytokine release, leading to a susceptibility to autoimmune diseases.<sup>27,76,77</sup> Early works have shown that peripheral injection of recombinant leptin improves T cell-mediated immune responses after fasting. Whereas central leptin treatment—using a recombinant adeno-associated virus (rAAV) system to deliver leptin gene directly into rat brain—reduced circulating leptin levels and body fat, as well as T cell-mediated delayed-type hypersensitivity (DTH) response. This was an indicative that leptin has a direct effect on immune cells that does not require adipose tissue signaling.<sup>78,79</sup>

## 5 | LEPTIN AND THE ADIPOSE TISSUE

Leptin discovery was an important breakthrough regarding the function of adipose tissue, now defined as an indispensable immunoenocrine organ.<sup>80–82</sup> Although the levels of leptin are correlated to the fat mass, its fluctuations accompany the energy status of the organism in a long-term fashion, increasing with energy surplus and decreasing during low energy periods such as fasting or cold exposure.<sup>83,84</sup> It is known that the different depots of white adipose tissue have different biology and functions, with increments in the visceral adipose

tissue (VAT) being strongly linked to adipocyte dysfunction, insulin resistance, and metabolic syndrome.<sup>85,86</sup> On the other hand, subcutaneous adipose tissue (SAT, or also known as SQ WAT) is recognized for its greater capacity to store lipids and its contribution to proper glucose homeostasis.<sup>87</sup> SAT buffers the excess fat, which otherwise would be toxic if in the circulation and accumulated ectopically or in VAT.<sup>88</sup> Consistently, insulin resistance is associated with lower levels of circulating leptin and reduced adipogenic capacity in the SAT of obese individuals, compared to insulin sensitive subjects.<sup>89</sup> Among adipose tissue depots, SAT is also the main source of leptin in humans<sup>86,90</sup> and it is tempting to speculate whether the role of leptin in this fat depot is beneficial or detrimental. In addition, the concentration of leptin in the adipose tissue microenvironment may be even higher than its serum levels, but this question remains to be addressed. Nevertheless, leptin autocrine and paracrine actions in adipocytes remain so far unexplored, although many studies have demonstrated the impact of leptin central signaling in the regulation of adipocyte physiology.<sup>91,92</sup>

A few studies aimed to determine the local effects of leptin in adipose tissues and adipocytes.<sup>93–95</sup> For example, treatment of epididymal WAT explants from rats with leptin for 1–2 h resulted in increased lipolysis.<sup>93,95</sup> However, in these cases, it is difficult to assume a direct effect of leptin on adipocytes because, as aforementioned, WAT is composed of a variety of cell types with M $\phi$ s making up for up to 50% of the total cell number in adipose tissue during obesity.<sup>96,97</sup> Besides, it is known that leptin activates immune cells and induces the production of TNF- $\alpha$ ,<sup>98</sup> a known lipolytic factor,<sup>99–102</sup> which favors a proinflammatory and prolipolytic milieu in the WAT (Fig. 2).<sup>98–102</sup> Importantly, TNF- $\alpha$  together with other adipokines and cytokines from resident and

recruited immune cells compose the adipose tissue microenvironment, which can modulate adipogenesis and proliferation of adipocyte precursor cells.<sup>103</sup> In another study, adipocytes were isolated from rat subcutaneous, epididymal, and perirenal adipose tissue and then stimulated with leptin for 2 h.<sup>94</sup> The authors concluded that leptin induced lipolysis in cells from these 3 fat depots by measuring the levels of secreted glycerol, a metabolite from the breakdown of stored triacylglycerol.<sup>94</sup> They also inferred that the optimal dose, with the strongest stimulation of lipolysis, was  $10^{-6}$  M (16,000 ng/mL) of leptin, which corresponds to more than 300 times the plasma levels of leptin in obese humans.<sup>90</sup> On the other hand, a recent study performed by Yue and colleagues showed that ObR specific deletion in the bone marrow stromal cells induced osteogenic differentiation at the expense of adipogenesis in the bone marrow of mice.<sup>104</sup> Also, they showed that the deletion reduced the total number of adipocytes and increased bone volume in high fat diet-fed mice, compared to DIO wild-type animals. These results suggest that leptin directly signals in stromal cells, being essential for their commitment with the adipocyte fate and for their proper differentiation.<sup>104</sup> Although this work increased our comprehension on the local effects of leptin in adipocytes and in precursor cell fate, many studies are still necessary to puzzle out the mechanisms of action of leptin and its roles in the direct modulation of adipogenesis and/or lipolysis. We cannot exclude the possibility that leptin may induce adipogenesis on precursor cells and lipolysis on mature adipocytes, but new studies are still necessary to unravel the resulting effect of physiological leptin concentrations on cells at different stages of adipocyte differentiation.

Although the above-mentioned evidences for a local proadipogenic effect of leptin in precursor cells and adipocytes, leptin signaling in nervous cells is known to induce energy expenditure through the modulation of glucose homeostasis and lipid metabolism.<sup>91</sup> For instance, Zeng and colleagues showed that adipocytes are surrounded by sympathetic fibers, creating what they called “neuro-adipose junctions,” which directly induce adipocyte lipolysis via peripheral neuronal stimulation.<sup>92</sup> Thus, leptin seems to have different actions in adipocytes depending on the type of signaling (local or neuronal). It is tempting to speculate that, during obesity, with the development of leptin central resistance, the peripheral effects of leptin on inflammatory and precursor cells may prevail. Nevertheless, how exactly these 2 signaling branches (lipolysis and lipogenesis/adipogenesis) are in harmony is definitely a question worthy of enlightenment.

Regarding brown adipose tissue, leptin increases the expression of uncoupling protein 1 (UCP1), one of the key proteins that mediate non-shivering thermogenesis.<sup>105</sup> Although leptin-deficient mice have lower body temperature and are hypothermic under low temperatures, the induction of thermogenesis by leptin is still controversial as the role of leptin in thermoregulation is linked to reductions in thermal conductance.<sup>106</sup> This places leptin as an essential factor for heat conservation, rather than heat generation (thermogenesis) itself.<sup>106</sup> Even though brown adipose tissue metabolism is a very interesting and wide issue, it is well discussed elsewhere<sup>91,107</sup> and is not in the scope of this review.

In summary, leptin signals in adipocytes, precursor cells, as well as in immune cells toward a complex equilibrium of metabolic and inflammatory pathways yet to be fully comprehended. In the growing WAT, leptin signaling together with other adipokines and cytokines, induces the recruitment and activation of immune cells, contributing to a proinflammatory milieu and the consequent release of free fatty acids that exacerbate obesity-related adipose tissue inflammation, in which M $\phi$ s play a major role.<sup>108</sup>

## 6 | RESIDENT M $\phi$ S

M $\phi$ s were first described by Elie Metchnikoff in 1892 as a phagocytic system.<sup>109</sup> Later, in 1968, van Furth and Cohn described several populations of M $\phi$ s originated from blood monocytes.<sup>110</sup> A study using blood monocytes-depleted mice showed that M $\phi$ s were able to maintain local proliferation in various tissues.<sup>111</sup> This discovery was corroborated by many other studies later in the 20th century showing the presence of tissue resident M $\phi$ s.<sup>112–116</sup> These M $\phi$ s had a distinct origin during embryonic development and were not derived from the bone marrow. Unlike blood monocytes that migrate to the tissue, resident M $\phi$ s are originated in the yolk sac and fetal liver and maintain the homeostasis of the tissue.<sup>117</sup> This was an important breakthrough, considering all the different functions of tissue resident M $\phi$ s in health and disease that we know of today. Kupffer cells (KCs) in the liver, microglia in the brain and osteoclasts in the bone are examples of resident M $\phi$ s with major roles in tissue immune surveillance.<sup>117</sup>

## 7 | M $\phi$ POLARIZATION

M $\phi$ s are important mediators of the innate immunity, with significant roles at keeping tissue homeostasis and fighting pathogens. M $\phi$ s produce numerous types of cytokines, which mediate pro- or anti-inflammatory responses upon specific stimuli.<sup>118</sup> M $\phi$ s are very plastic cells and can undergo polarization toward distinct profiles.<sup>119–123</sup> There is a large variation of M $\phi$  phenotypes, but for didactic reasons we use a well-accepted model with the populations that are best described.<sup>124–128</sup> There are 2 populations of M $\phi$ s that were extensively studied and are well characterized. The classically activated M $\phi$ s, or M1, which are induced by TLR4 activation and IFN- $\gamma$ .<sup>129</sup> M1 M $\phi$ s have a proinflammatory pattern, with high phagocytic activity and intense production of bactericidal mediators, due to large expression of nitric oxide synthase (iNOS) and reactive oxygen species (ROS) generation.<sup>15,129</sup> The alternatively activated M $\phi$ s, or M2, are induced by interleukin 4 (IL-4) and mediate responses against helminths. M2 M $\phi$ s also participate in the resolution phase of inflammation and tissue repair, express high levels of arginase-1 (ARG-1) and secrete the anti-inflammatory cytokine IL-10.<sup>129,130</sup> The M1\M2 characterization was proposed by Mills and collaborators in view of the adopted model for T helper lymphocytes. Once activated by IL-12, T helper 1 (Th1) cells produce large amounts of IFN- $\gamma$ . Whereas Th2 cells, which are induced by IL-4, secrete Th2 types of cytokines, such as

IL-4 and IL-13. The balance between M1/M2 responses is crucial for systemic homeostasis.<sup>118,131</sup>

## 8 | M $\phi$ METABOLISM—AN OVERVIEW

M1 and M2 M $\phi$ s execute different functions with distinctive immunologic profiles. In addition, M1 and M2 M $\phi$ s are also distinctive metabolically. In the last decade, a large number of studies showed that M $\phi$ s when activated shift their metabolism from oxidative phosphorylation to aerobic glycolysis.<sup>127,129,132</sup> This change in intracellular metabolism also happens in tumor cells.<sup>133</sup> This phenomenon is called the Warburg Effect.<sup>134</sup> It was first described in the 1920s during cancer studies.<sup>134</sup> The Warburg Effect consists on a shift to glycolysis by tumor cells, even when oxygen is available for ATP generation. This is an important feature of tumor cells metabolism, because relying on glycolysis means fast ATP production (even though the final ATP count of glycolysis is much lower than oxidative metabolism in the mitochondria, the reaction occurs up to 100 times faster).<sup>135</sup> Cancer cells proliferate at abnormally high rates and the increased glucose uptake fuels the glycolytic flux providing carbon sources for anabolic processes necessary for the biosynthesis of molecules and cell proliferation.<sup>133,136</sup> The changes in intracellular metabolism, with enhanced glucose uptake are accompanied by mitochondrial remodeling, with accumulation of dysfunctional mitochondria, which have diminished respiratory capacity.<sup>136,137</sup>

Immune cells undergo similar metabolic changes upon activation and engage on what is called aerobic glycolysis. Activated and pro-inflammatory lymphocytes, dendritic cells, and M $\phi$ s perform aerobic glycolysis instead of oxidative respiration and accumulate dysfunctional, nonrespiratory mitochondria even during normoxia.<sup>127,128,138–142</sup> Metabolic reprogramming is a critical process, which directly influences M $\phi$  function. M $\phi$ s need to promote fast responses that require intense protein and lipid synthesis to support the production of antibacterial and inflammatory mediators, such as proinflammatory cytokines, NO, and ROS.<sup>127,143</sup> Enhanced glycolysis has become one of the signatures of the pro-inflammatory activity by M $\phi$ s.<sup>127</sup> Increased levels of glucose transporter 1 (GLUT1), as well as determinant enzymes in the glycolytic flux—such as 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), pyruvate kinase M2 (PKM2), and lactate dehydrogenase (LDH)—are associated with higher rates of proinflammatory cytokine expression.<sup>127,144</sup> The importance of glycolysis on M $\phi$  inflammatory function was determined by different studies that showed elevated cytokine expression and phagocytic activity by glycolytic M $\phi$ s.<sup>145,146</sup>

The proinflammatory profile has been extensively associated with the metabolic shift to glycolysis and accumulation of dysfunctional mitochondria.<sup>138,140,147</sup> These are characteristics observed in lipopolysaccharide (LPS)-activated M $\phi$ s, and also in polarized M1 M $\phi$ s. TLR4 activation by LPS activates the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), a transcription factor induced by hypoxic conditions, responsible for the induction of glycolysis and the expression of IL-1 $\beta$  in M $\phi$ s.<sup>148</sup> This all means that when M $\phi$ s leave the quiescent state, they

undergo intense metabolic reprogramming to feed their new immunologic demands.

Unlike the proinflammatory M $\phi$ s, M2 M $\phi$ s present higher oxygen consumption rates, with the absence of dysfunctional mitochondria.<sup>149</sup> M2 M $\phi$ s have enhanced activity of PPAR $\gamma$ -coactivator-1 $\beta$  (PGC-1 $\beta$ ) and signal transducer and activator of transcription 6 (STAT6), 2 important transcription factors, which are induced by IL-4 and greatly inhibit the production of proinflammatory cytokines.<sup>150</sup> Another important feature of M2 M $\phi$ s is the increased expression of IL-10, a known anti-inflammatory cytokine. IL-10 contributes to M $\phi$  metabolic modulation, by inhibiting the mammalian target of rapamycin (mTOR).<sup>140</sup> mTOR is a key intracellular metabolic sensor, with almost antagonizing roles from AMP-activated protein kinase (AMPK).<sup>141</sup> AMPK is an inhibitor of mTOR and can activate mitophagy, which is essential for the maintenance of respiratory mitochondria for oxidative metabolism.<sup>151–153</sup> The complex 1 of mTOR (mTORC1) increases glycolysis and dysfunctional mitochondria in different cell types.<sup>153,154</sup> On the other hand, the complex 2 of mTOR (mTORC2) is essential for M2 activity in IL-4-stimulated M $\phi$ s. Parallel induction of STAT6 by IL-4 receptor and mTORC2 leads to IRF4 expression and M2 response.<sup>124</sup> The role of mTORC1 and mTORC2 in M $\phi$  immunometabolism is not yet fully understood, but the activation of either complex seems to induce opposite responses in M $\phi$ s.

Until very recently, fatty acid oxidation (FAO) was thought to be an essential step for M2 polarization. However, a growing number of studies showed that, even though FAO and the expression of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ )—a nuclear receptor that controls the expression of lipid metabolism-related genes—are increased in M2 M $\phi$ s, FAO is not required for M2 polarization.<sup>155,156</sup> In fact, IL-4 stimulated M $\phi$ s boost glucose utilization and that is an essential step for M2 polarization.<sup>124</sup> Increased FAO, however, is not an essential step for M2 polarization. M $\phi$ s treated with low concentrations ( $\geq 10$   $\mu$ M) of etomoxir, an inhibitor of carnitine palmitoyltransferase-1 (CPT-1)—a mitochondrial enzyme necessary for FAO, did not show reduced M2 polarization.<sup>155</sup> This concentration is enough to block CPT-1 activity.<sup>155</sup> Earlier studies utilized a high dose of 200  $\mu$ M of etomoxir, which disturbs intracellular coenzyme A (CoA) homeostasis and inhibits M2 polarization.<sup>155,156</sup> M $\phi$ s can present different metabolic profiles depending on where they are located and which roles they are supposed to engage.<sup>111,147</sup> The patterns of metabolic behavior in resident M $\phi$ s result in distinct immune profiles, which are important to attend specific tissue necessities.

## 9 | ADIPOSE TISSUE RESIDENT M $\phi$ S IN OBESITY

The larger leukocyte population in the adipose tissue are the ATMs and the number of ATMs increases with adiposity, going from  $\sim 15\%$  in lean mice up to  $\sim 50\%$  in obese mice.<sup>97,157</sup> The majority of the M $\phi$ s in lean adipose tissue are characteristically M2-like, which

means they express classic M2 markers, such as the surface markers CD206, CD301, CD163, and other genes that promote anti-inflammatory protein transcription, including ARG-1 and IL-10.<sup>15</sup> These M2-like M $\phi$ s are responsible for tissue maintenance and present anti-inflammatory aspects.<sup>14,15,158,159</sup>

In obese individuals, there is an unbalance in the proportion of M2-/M1-like ATMs. This switch in ATM phenotype is caused by the infiltration of pro-inflammatory M $\phi$ s, which disrupts adipose tissue homeostasis and leads to inflammation and insulin resistance (Fig. 2).<sup>14</sup> Besides that obese adipose tissue creates a hypoxic environment, which forces the resident M $\phi$ s to adapt and change their core metabolism.<sup>160</sup> Other obesity-associated factors, such as adipogenesis and insulin induce the up-regulation of HIF-1 $\alpha$  mRNA and protein levels, which promote tissue inflammatory response.<sup>161–164</sup> In the same way, deletion of HIF-1 $\alpha$  in M $\phi$ s protects mice from HFD-induced inflammation, by reducing the formation of hypoxic “crown-like” structures and inducing the expression of angiogenic factors that are necessary for adipose tissue physiological remodeling.<sup>165</sup>

Adipocytes express monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), which has high affinity with CCR2.<sup>166,167</sup> This is the mechanism by which CCR2 circulating monocytes are recruited to the adipose tissue and differentiate into proinflammatory M $\phi$ s.<sup>166,167</sup> In obesity, MCP-1 levels are elevated in the adipose tissue.<sup>168</sup> When overexpressed in the adipose tissue, MCP-1 leads to exacerbated M $\phi$  recruitment and insulin resistance.<sup>169,170</sup> On the other hand, CCR2 deficiency in mice protects against diet induced insulin resistance.<sup>168</sup> This does not mean that the number of M2-like ATMs is reduced in obesity. Obese adipose tissue present increased total cell number—including M $\phi$ s, and the number of M2-like M $\phi$ s remains the highest. However, the proportion in the M1/M2 rate is altered, with an increase in the percentage of M1-like proinflammatory M $\phi$ s.<sup>159</sup>

CD11c is an integrin that is expressed in DCs, M $\phi$ s, and even B cells.<sup>171,172</sup> In cases of obesity, proinflammatory CD11c<sup>+</sup> M $\phi$ s accumulate in the adipose tissue, forming “crown-like” structures around necrotic adipocytes.<sup>173,174</sup> CD11c<sup>+</sup> ATMs show increased proinflammatory features, including decreased IL-10 expression and high proinflammatory cytokines production (IL-6, TNF, IL-1 $\beta$ ) and iNOS expression. The M2-like ATMs are identified as F4/80<sup>+</sup>CD206<sup>+</sup>CD11c<sup>-</sup>.<sup>14,159,171</sup> Although human ATMs are of an anti-inflammatory phenotype, they are able to induce several proinflammatory mediators, being characterized as F4/80<sup>+</sup>CD206<sup>+</sup>CD11c<sup>+</sup>. This means that the M2-like M $\phi$ s conserve their main function in the tissue, while also being able to secrete proinflammatory cytokines and chemokines.<sup>175,176</sup> This sort of M2 M $\phi$  with proinflammatory roles has been recently studied in different contexts also in mice.<sup>126</sup> Another important aspect of CD11c<sup>+</sup> M $\phi$ s is the fact that they can impair glucose uptake by adipocytes and are a major contributor to insulin resistance.<sup>174</sup>

Aside from M $\phi$ s, T cells in fat depots help maintain tissue homeostasis. Obesogenic conditions increase the number and frequency of proinflammatory M $\phi$ s and effector T cells, including both CD4 Th cells and CD8 cytotoxic T cells.<sup>177–179</sup> In contrast, immune cells with

regulatory profiles and cells associated with type 2 immune responses, such as type 2 innate lymphoid cells (ILC2), invariant natural killer cells (iNKT) and regulatory T cells are reduced in obese individuals.<sup>180–182</sup> Metabolic disorders and adipose tissue inflammation are associated with this disruption in adipose tissue homeostasis, which strongly relies on type 2 immune cells regulation and repair.<sup>182</sup> Leptin suppresses regulatory T cell differentiation and is a key regulator of T effector cells differentiation, such as Th17. Leptin is necessary for HIF-1 $\alpha$  expression, which is important for the up-regulation of glycolytic metabolism in effector T cells.<sup>183</sup>

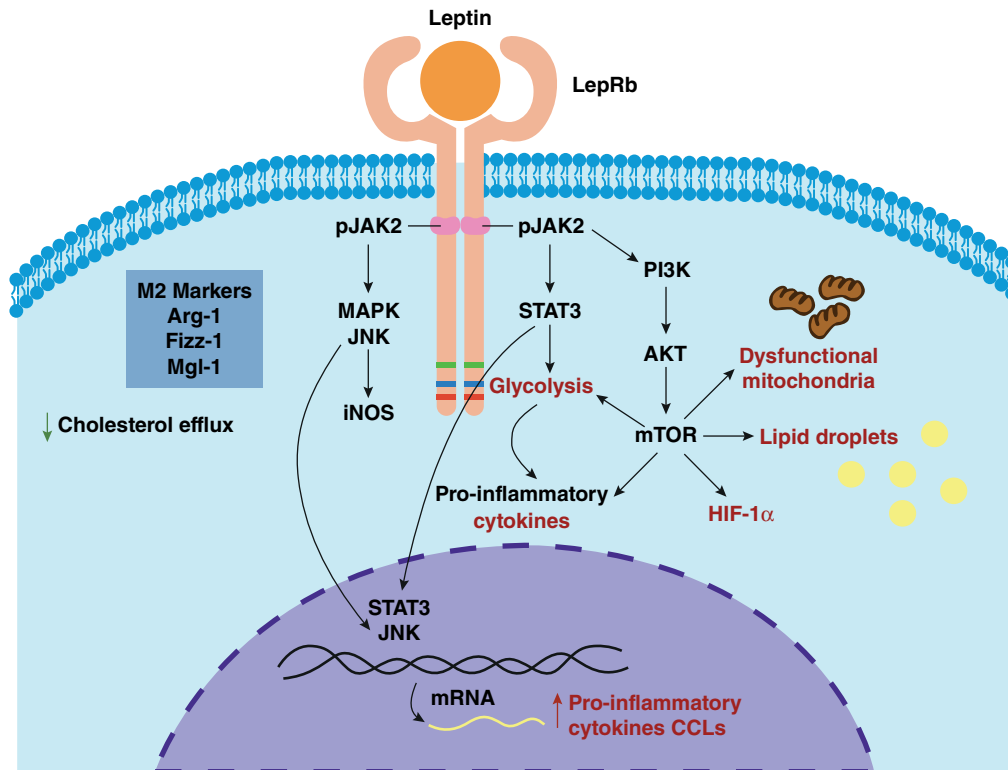
WAT inflammation is associated with activation of immune cells, the presence of enlarged, unhealthy adipocytes and the accumulation of collagen fibers in the tissue. A recent study identified a new inflammatory subpopulation of cells in the adipose tissue of mice.<sup>184</sup> A combined technique of single-cell RNA-sequencing and FACS isolation was utilized to identify the so called fibro-inflammatory progenitors (FIPs) cells residing around the blood vessels in the visceral adipose tissue of mice.<sup>184,185</sup> FIPs can activate immune cells in WAT and contribute to WAT remodeling and fibrosis.<sup>184</sup>

## 10 | LEPTIN AND M $\phi$ IMMUNOREGULATION

The role of leptin as inductor of M $\phi$  proinflammatory immune response has been reviewed in different works.<sup>186,187</sup> The functional isoform of Ob-Rb is not expressed only in the hypothalamus, but also in several cell types of innate and adaptive immunity.<sup>25,186–191</sup> In the immune system, leptin activates proinflammatory cells, promoting Th1 types of response, and mediating the production of the other proinflammatory cytokines, such as TNF- $\alpha$ , IL-12, or IL-6.<sup>187,192,193</sup> Conversely, leptin receptor is also up-regulated by proinflammatory signals.<sup>194</sup> Leptin is also an important mediator of autoimmune conditions.<sup>193</sup> Matarese and colleagues showed that ob/ob mice are protected from experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis.<sup>77</sup> Another study by Siegmund and collaborators described how ob/ob mice are resistant to acute and chronic intestinal inflammation induced by dextran sodium sulphate and to colitis induced by trinitrobenzene sulphonic acid (experimentally induced colitis, EIC). They showed that CD8<sup>+</sup> intraepithelial lymphocytes had reduced capacity of IFN- $\gamma$  production and were also significantly reduced in number.<sup>195</sup> There are several studies linking leptin to lymphocyte-dependent responses. Leptin deficiency results in enhanced Th2 responses and allograft survival and impairs Th17 differentiation.<sup>78,196,197</sup> However, the role of leptin in innate immunity has not been as extensively investigated.

The activation of the long isoform of the human leptin receptor in PBMCs can lead to the activation of 2 signaling pathways, PI3K and mitogen-activated protein kinase (MAPK).<sup>188</sup> This is also true in M $\phi$ s, since leptin treatment resulted in enhanced TNF- $\alpha$  production accompanied by a dose-dependent increase of MAPK activity in LPS-stimulated Kupffer cells.<sup>190</sup> MAPKs are a family of proinflammatory proteins that can be activated by LPS and induce the release of inflammatory mediators by M $\phi$ s.<sup>198</sup> Besides cytokine production,





**FIGURE 3** Mechanisms of leptin-induced immunometabolic changes in M $\phi$ s. Leptin signals through LepRb, the functional isoform of the leptin receptors, which is expressed by M $\phi$ s. The phenotype presented by M $\phi$ s upon leptin treatment resembles a proinflammatory M2 M $\phi$ . Leptin leads to upregulation of typical M2 markers, such as ARG-1, FIZZ-1, and MGL-1, while also activating proinflammatory MAPKs. Activation of JNK by leptin results in up-regulation of iNOS and proinflammatory cytokines. JAK2-STAT3 activation that occurs after leptin treatment leads to the migration of phosphorylated STAT3 to the nucleus, where it promotes the expression of CCLs. STAT3 also increases the expression of enzymes that regulate glycolytic flux. Leptin also activates the PI3K-AKT-mTOR pathway, thereby increasing glycolysis, HIF-1 $\alpha$  expression, and consequently, proinflammatory cytokine expression. mTOR activation by leptin leads to lipid droplets formation and reduced cholesterol efflux in M $\phi$ s. mTOR also contributes to the accumulation of dysfunctional mitochondria in M $\phi$ s, by inhibiting mitophagy and disrupting homeostatic mitochondrial remodelling

leptin also up-regulates phagocytic function in M $\phi$ s.<sup>199</sup> In human M $\phi$ s, leptin enhances CCLs expression, via activation of the canonical JAK2-STAT3 pathway and potentiates LPS response (Fig. 3).<sup>200,201</sup>

Obesity leads to exacerbated leptin release by the adipose tissue. In obese mice, saturated free fatty acids (FFAs) produce a pro-inflammatory M $\phi$  phenotype that is mechanistically distinct from M1 or M2 activation.<sup>202,203</sup> During differentiation of monocytes into M $\phi$ s, Ob-Rb is up-regulated and maintained after differentiation, providing stronger responses to leptin by M $\phi$ s.<sup>204</sup> Leptin up-regulates Acetyl-CoA acetyltransferase (ACAT-1) expression via PI3K-JAK2 signaling in human M $\phi$ s.<sup>204</sup> ACAT-1 is a mitochondrial protein that induces cholesteryl ester formation from cholesterol. Thus, leptin accelerates cholesteryl ester accumulation within the cell, thereby suppressing cholesterol efflux.<sup>204</sup> Besides, leptin can activate the mTOR pathway via PI3K signaling in M $\phi$ s (Fig. 3).<sup>60</sup> A study by Maya-Monteiro and colleagues showed that leptin induces the activation of mTORC1, leading to the formation of lipid droplets. The newly formed lipid bodies were sites of 5-lipoxygenase localization and correlated with an enhanced capacity of leukotriene B4 production, inducing an inflammatory phenotype.<sup>59</sup>

mTOR activation by leptin in M $\phi$ s is a clear indicative of metabolic modulation, since mTOR is not only involved in lipid metabolism, but can also induce glycolysis and mitochondrial remodeling.<sup>60,153</sup> The effects of leptin in the metabolism of M $\phi$ s still not known. However, metabolic modulation by leptin has been recently described in tilapia (*Oreochromis mossambicus*) pituitary.<sup>205</sup> A study demonstrated through transcriptome analysis that leptin regulates PFKFB3, a critical stimulator of phosphofructokinase (PFK), the rate limiting enzyme for glycolysis, and the glycolytic enzyme GAPDH. Both PFK activity and mRNA expression are correlated with lactate production, the final product of the glycolytic pathway. They also showed that leptin up-regulates HIF-1 $\alpha$  mRNA levels. Besides that, constitutive STAT3 activation leads to higher rates of glycolysis. In addition, inhibition of STAT3 resulted in the suppression of these responses, indicating that leptin stimulates glycolysis through a STAT3-dependent mechanism.<sup>205</sup> The metabolic phenotype observed after leptin treatment resembles the metabolic switch by which M $\phi$ s undergo upon activation. The microenvironment in obese adipose tissue retains higher levels of leptin secreted by adipocytes. Adipose tissue resident M $\phi$ s are exposed to the excessive contact of with leptin in the obese adipose tissue. This could be an

important step to take in the study of M $\phi$  metabolic reprogramming and obesity-induced inflammation.

## 11 | LEPTIN IN ADIPOSE TISSUE M $\phi$ IMMUNOMETABOLIC MODULATION

Exacerbated TNF- $\alpha$  concentration is a trait observed in several obesity-associated conditions, such as T2D, cardiovascular diseases, and inflammation.<sup>3,14,31,159,206</sup> This is closely related to obesity morbidity and mortality.<sup>6,207</sup> Excessive cytokine secretion during these cases rely on altered M $\phi$  activity in the adipose tissue.<sup>97,208</sup> ATMs are in constant contact with secreted leptin in the adipose tissue environment and can be directly affected when leptin levels are unbalanced. The immunoregulatory effects of leptin were first noticed during studies in rodents with genetic abnormalities in leptin or leptin receptor that became obese at a young age, now known as ob/ob and db/db.<sup>209–212</sup> M $\phi$ s from mice with impaired leptin signaling have deficient phagocytic activity and proinflammatory cytokine production and treatment with exogenous leptin ameliorates M $\phi$  function in ob/ob animals.<sup>213</sup> Leptin treatment in ob/ob female mice also improved insulin resistance while inducing M2 markers expression (Fig. 3) (ARG-1, Fizz-1, Mgl1/2).<sup>214</sup> Human M $\phi$ s respond similarly to leptin treatment.<sup>175</sup> CD14 human M $\phi$ s were isolated and treated with leptin or polarized in culture toward an M1 phenotype (with IFN- $\gamma$ ) or an M2 phenotype (with IL-4). When treated with leptin in culture, M $\phi$ s also up-regulated the expression of typical M2 markers, while being able to secrete high levels of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-1ra, IL-10, MCP-1, and MIP-1 $\alpha$ , which are characteristic for M1 cells.<sup>175</sup> This suggests that leptin is a strong contributor to the distinct phenotype observed in ATMs. The production of these inflammatory factors is under the transcriptional control of JNK—a key inflammatory protein in the MAPK family, and NF- $\kappa$ B. Both JNK and NF- $\kappa$ B are significantly up-regulated in the adipose tissue.<sup>97,215</sup> Both inducers of the inflammatory response differ in their upstream signaling components. However, previous studies showed that leptin induces the activation of MAPKs as one of its inflammatory mechanisms.<sup>11,188,190</sup> Both JNK and NF- $\kappa$ B pathways are induced by mediators that have been implicated in the occurrence of insulin resistance, such as FFAs, proinflammatory cytokines, oxidative stress, and endoplasmic reticulum stress.<sup>216</sup> Leptin signaling in ATMs leads to the activation of pathways linked to the pathogenesis of different metabolic regulated diseases.

Human leptin stimulates M $\phi$  proliferation in a dose-dependent manner, meaning hyperleptinemic conditions can induce ATM proliferation. Leptin also activates circulating monocytes and induces the secretion of TNF- $\alpha$  and IL-6 and the expression of activation surface markers, such as CD25, HLA-DR, CD38, CD71, CD11b, and CD11c.<sup>217</sup> Besides that, leptin induces the expression of adhesion molecules in the endothelial cells of the stromal vascular compartment, which can increase M $\phi$  infiltration to the adipose tissue.<sup>208,218</sup> The enhanced levels of leptin secreted in the obese adipose tissue is, in part, responsible for the unbalanced accumulation and activation of M $\phi$ s in the adipose tissue. These M $\phi$ s have a distinct phenotype, being

characteristically M2-like with high proinflammatory features, which are accompanied by a switch in cell core metabolism.

## 12 | CONCLUDING REMARKS

During obesity, leptin levels are significantly increased and lead to a disruption in physiological function.<sup>219</sup> M $\phi$  metabolic reprogramming has been the target of studies from different perspectives. The metabolic switch to glycolysis is a marker of proinflammatory signaling, which is marked by up-regulation of glycolytic enzymes, such as GLUT1, PFKFB3, PKM2, and LDH. Other changes include accumulation of dysfunctional mitochondria with reduced respiratory capacity and induction of metabolic regulators, such as mTOR and HIF-1 $\alpha$ . ATMs are phenotypically close to M2 M $\phi$ s and maintain adipose tissue homeostasis. Both adipocytes and ATMs can be modulated by excessive leptin concentrations, resulting in proinflammatory responses. Leptin can activate different intracellular pathways, derived from STAT3 or PI3K activation. Both signals can induce metabolic and functional changes in M $\phi$ s. Leptin promotes proinflammatory cytokines release and lipid body formation, as well as activate factors related to glycolytic switch. Obesity is a multifactorial disease, which complicates the induction of a drug with specific targets to ameliorate obesity-related low-grade chronic inflammation. Leptin signaling is a key factor to be considered as a therapeutic target, due to its many roles in M $\phi$  modulation. In this review, we regarded the importance of leptin in obese conditions by exploring different works focused on immunologic and metabolic approaches.

### AUTHORSHIP

L.M., J.A.S.P., and L.P. wrote the manuscript and made figures. P.M.M.M.-V. edited and revised the manuscript.

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### DISCLOSURE

The authors declare no conflict of interest.

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## **APENDICE 4**



## Main Manuscript for

# Activation of liver X receptor $\beta$ reduces adipose tissue inflammation and improves insulin sensitivity during obesity

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**Author Contributions:** JASP designed and performed experiments, analyzed data, wrote the manuscript and made the figures. GPS, JVV, JSP, ACC, GC, LFP, FMN, GGD, CFA, CNS, MG performed experiments. HN performed the bioinformatic analysis. RCA, MASM, ASB, NOSC provided equipment, reagents and expertise. PMMMMV coordinated the project, designed experiments, wrote and edited the manuscript.

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**This PDF file includes:**

Main Text

Figures 1 to 4

## Abstract

Liver X receptors (LXR) regulates cholesterol homeostasis and immune response. Cholesterol levels are commonly increased in obesity and contribute for pro-inflammatory responses. The role of LXR in obesity-induced adipose tissue (AT) inflammation, macrophage phenotype and glucose homeostasis remain to be elucidated. Here, we show that LXR deletion boosts AT inflammation in an LXR $\beta$ -dependent manner. LXR deficiency in immune cells aggravates obesity-induced AT inflammation by increasing CD11c<sup>+</sup> and IL-1 $\beta$ <sup>+</sup> AT macrophage (ATM) numbers. Finally, we identified naringenin (NAR) as an LXR agonist in macrophages. NAR treatment reduced pro-inflammatory cytokines expression in activated macrophages. Obese mice treated with NAR displayed increased insulin sensitivity and reduced AT inflammation. Together, we show a distinct role for each LXR isoform in the regulation of AT inflammation and glucose homeostasis. LXR $\beta$  expression in immune cells is necessary for AT pro/anti-inflammatory balance. LXR $\beta$  has the potential to become a therapeutic target to treat AT inflammation and insulin resistance.

## Significance Statement

Obesity has reached epidemic rates and contributes for the development of many other disorders, as cardiovascular disease and diabetes. A shift in the resident adipose tissue macrophage population toward a pro-inflammatory profile is frequently found in obese people. However, how obesity affects nuclear receptors and AT macrophages function is still unknown. Here we show that ATMs from obese animals have enriched cholesterol pathway, and LXR deletion boost the lipid accumulation in macrophages, while increase the adipose tissue inflammation in a LXR $\beta$ -dependent manner, while LXR $\beta$  activation increase insulin sensitivity by reducing adipose tissue inflammation.

## Main Text

### Introduction

Liver X receptors (LXR) are transcriptional factors that act as cholesterol sensors and regulate the efflux, degradation and transport of cholesterol, through the expression of ATP binding cassette (ABC) transporters, apolipoproteins and other genes related to the reverse cholesterol transport (1-3). LXR activation occurs in response to endogenous ligands, such as oxysterols (4-6), or synthetic molecules, such as T0901317 and GW3965 (7, 8). LXR also play a role in glucose homeostasis and carbohydrate metabolism by regulating the expression of glucose transporter 4 (GLUT4) and controlling insulin secretion and hepatic glucose metabolism (5, 9-12). LXR have two isoforms, LXR $\alpha$  and LXR $\beta$ , which have high homology but distinct expression patterns (3, 13). LXR $\alpha$  expression is restricted to visceral organs and immune cells, such as macrophages (14, 15)(16), while LXR $\beta$  is ubiquitously expressed (3).

Obesity is marked by chronic low-grade inflammation (17, 18). Obese people commonly display increased cholesterol and glucose levels, which can affect LXR expression and activation (19). Furthermore, obesity-induced AT inflammation contributes to impaired insulin sensitivity, which is a major risk factor for the development of type 2 diabetes (20). Several immune cell types participate in the regulation of AT inflammation and insulin sensitivity (21). In lean mice and humans, macrophages maintain AT homeostasis (22). Obesity induces the accumulation of inflammatory macrophages and other pro-inflammatory immune cells, leading to AT inflammation and the subsequent insulin resistance (23).

Adipose tissue macrophages (ATM) span a constellation of phenotypes and obesity triggers phenotypic changes in ATM populations (24). Simplistically, ATM phenotypes are usually divided in a tissue maintenance/anti-inflammatory phenotype and a pro-inflammatory phenotype (20, 25-28). Alternatively activated ATM numbers are higher in lean AT and these macrophages express CD206 (mannose receptor), macrophage galactose-type lectin (MGL), transforming growth factor- $\beta$  (TGF- $\beta$ ), IL-10, among others, and thus, contribute to AT homeostasis and repair (29-31). Classically activated ATM express CD11c (integrin alpha X) and pro-inflammatory cytokines and chemokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and CCL-2 and iNOS (26, 32-38). Obesity induces an imbalance between CD206<sup>+</sup> and CD11c<sup>+</sup> macrophages, which contribute to the development of insulin resistance, type 2 diabetes and atherosclerosis in obese subjects (39).

1 Several studies have attributed an anti-inflammatory role for LXR (40-42). Agonists of LXR inhibit  
2 the activation of NF $\kappa$ B, as well the expression of pro-inflammatory genes in macrophages (3, 43,  
3 44). However, the role of each LXR isoform in macrophages and in the regulation of ATM  
4 metabolism and function during obesity remains unclear. Here, we show that the deletion of any  
5 LXR isoform results in immune imbalance in AT of lean animals. In response to high fat diet  
6 (HFD), only LXR $\beta$  deficiency aggravates obesity-induced AT inflammation. LXR $\alpha$  and  $\beta$ -deficient  
7 animals display increased numbers of IL-1 $\beta$ <sup>+</sup> ATM in the perigonadal AT (PgAT). Moreover, the  
8 deletion of both LXR isoforms specifically in immune cells is sufficient to alter AT immune  
9 phenotype, observed by increased monocyte infiltration and CD11c<sup>+</sup> ATM numbers in lean  
10 animals, and increased expression of pro-inflammatory cytokines in both lean and obese animals.  
11 Finally, we found that the citrus-derived flavonoid naringenin acts as a novel LXR $\beta$  agonist in  
12 macrophages. Naringenin treatment reduces the expression of pro-inflammatory markers by  
13 reducing LPS-induced glycolysis and mitochondrial dysfunction in macrophages. In vivo,  
14 naringenin-induced LXR $\beta$  activation in obese mice reduces the number of AT pro-inflammatory  
15 macrophages, mitigating insulin resistance.  
16

## 17 **Results**

### 18 ***LXR deficiency affects body weight gain and insulin sensitivity in HFD-fed mice***

19 Obesity is caused by the imbalance between energy intake and energy expenditure, which  
20 promotes the expansion of white adipose depots and increases circulating fatty acids and  
21 cholesterol levels (45). In turn, increased cholesterol levels may directly lead to LXR activation  
22 (4). Aiming to determine the effects of LXR during obesity, we analyzed the systemic metabolic  
23 effects of LXR $\alpha$  and  $\beta$  deletion (LXR $\alpha\beta$ KO) in mice fed a high fat diet (HFD) for 12 weeks. LXR  
24 deficiency inhibited HFD-induced weight gain and AT expansion (Fig. 1A and Fig. S1A) with no  
25 changes on cumulative food intake (Fig. 1B and Fig. S1B). Next, we determined the role of LXR  
26 in the regulation of glucose homeostasis under obesogenic conditions. HFD-fed LXR $\alpha\beta$ KO mice  
27 had no alterations in fasting blood glucose levels (Fig. 1C), but displayed increased insulin  
28 sensitivity and glucose intolerance when compared to HFD-fed wild-type (WT) controls (Fig. 1D-E  
29 and Fig. S1C). We next investigated if the reduced weight gain in HFD-fed LXR $\alpha\beta$ KO was  
30 associated with changes in energy expenditure. HFD-fed LXR $\alpha\beta$ KO mice displayed reduced  
31 oxygen consumption compared to WT animals in the same diet (Fig. 1F). In addition, the energy  
32 expenditure was reduced in LXR $\alpha\beta$ KO (Fig. S1D), with no changes in mice activity (Fig. S1E).  
33 We then explored whether LXR $\alpha\beta$ KO could affect fuel source for energy generation. LXR $\alpha\beta$ KO  
34 used glucose as the preferential fuel for energy production, while HFD-fed WT animals primarily  
35 used lipids (Fig. 1G-H). These data indicate that LXR are important regulators of energy balance  
36 and contribute to the maintenance of whole-body glucose homeostasis.  
37

### 38 ***LXR $\alpha$ deletion is sufficient to improve insulin sensitivity in obese mice***

39 LXR isoforms has been showed to induce distinct cellular response upon activation (46).  
40 However, whether LXR $\alpha$  and LXR $\beta$  may promote distinct effects over the whole-body metabolism  
41 is still unknown. We fed WT, LXR $\alpha$ KO and LXR $\beta$ KO mice with HFD and evaluated the effects of  
42 individual deletion of the LXR isoforms on HFD-induced weight gain and systemic glucose  
43 homeostasis. LXR $\alpha$ KO and LXR $\beta$ KO mice gained similar weight as WT controls (Fig. 1I). Also,  
44 neither LXR $\alpha$  nor LXR $\beta$  deletion promoted changes in food intake, fat mass and fasting glycemia  
45 (Fig. 1J-K and Fig. S1F). LXR $\alpha$ KO mice displayed increased insulin sensitivity under obesogenic  
46 conditions compared to WT controls and LXR $\beta$ KO mice (Fig. 1L). LXR $\beta$ KO presented impaired  
47 glucose tolerance compared to WT controls and LXR $\alpha$ KO mice (Fig. 1M). In order to verify  
48 whether LXR $\alpha$  or LXR $\beta$  deletion could affect energy expenditure and energy source under  
49 obesogenic conditions, indirect calorimetry analysis was performed. Both LXR $\alpha$ KO and LXR $\beta$ KO  
50 mice had similar oxygen consumption and energy expenditure as WT controls (Fig. 1N and Fig.  
51 S1G). Also, both LXR $\alpha$ KO and LXR $\beta$ KO mice displayed similar preference for lipids as fuel for  
52 energy production, similar to WT mice (Fig. 1O). Together, our data show that, albeit there is a  
53 high overlap between the transcriptional function of LXR $\alpha$  and LXR $\beta$  (13, 47), each LXR isoform  
54 displays a distinct role in the regulation of systemic glucose homeostasis.  
55

### 56 ***LXR is necessary for ATM inflammatory balance in lean white adipose tissue***

1 Obesity disturbs AT homeostasis, which includes not only the alteration in adipocyte size and  
2 morphology, but also comprises the switch from a reparatory into a pro-inflammatory profile in a  
3 variety of immune cells (18). Increased cholesterol levels activate pro-inflammatory pathways in  
4 immune cells (48). Also, obesity promotes metabolic adaptations in immune cells, such as ATM,  
5 directly affecting their function through a still not completely understood mechanism. Because  
6 macrophages are the most abundant immune cell population in AT of obese mice and humans  
7 (49), we first determined if the main metabolic pathways modulated by the disruption of AT  
8 homeostasis are related to cholesterol metabolism. We evaluated RNA-sequencing data from Pg  
9 ATM (F4/80<sup>+</sup>CD11b<sup>+</sup>) combining CD11c<sup>+</sup> and CD11c<sup>-</sup> cells in both lean and obese animals. We  
10 observed that among the most modulated pathways during obesity, such as the lysosome  
11 pathway, as previously described (50), cholesterol metabolism is upregulated in ATM from obese  
12 mice (Fig. 2A). Next, we analyzed the expression of genes related to cholesterol metabolism and  
13 found a marked increase in the expression of *Nr1h3* and *Nr1h2* (LXR $\alpha$  and LXR $\beta$ , respectively),  
14 and of LXR-target genes *Lpl*, *Abcg1* and *ApoE* (Fig. 2B). These data suggest that LXR may have  
15 a direct effect on ATM phenotype and function during obesity.

16 We hypothesized that LXR may directly impact ATM pro-inflammatory profile. We observed that  
17 the lack of both LXR isoforms in HFD-fed mice reduced the total number of ATM and the number  
18 of CD11c<sup>+</sup> and CD206<sup>+</sup> ATMs in PgAT in comparison to WT mice fed with the same diet (Fig. 2C  
19 and Fig. S2A). Despite the reduction in the total macrophage population, LXR $\alpha$  $\beta$ KO mice  
20 displayed a similar CD11c/CD206 ratio when compared to WT HFD-fed mice (Fig. 2C). Thus,  
21 LXR deficiency leads to a global reduction in macrophage population, affecting in a similar  
22 manner both CD11c<sup>+</sup> and CD206<sup>+</sup> ATM populations.

23 Despite the changes in ATM density in LXR $\alpha$  $\beta$ KO mice, we found that individual deletion of LXR  
24 isoforms did not affect the total number of perigonadal ATM compared to WT mice when  
25 subjected to HFD (Fig. 2D and Fig. S2B). We also evaluated ATM populations in chow-fed LXR  
26 single knockout mice and observed that chow-fed LXR $\alpha$ KO mice have increased number of ATM  
27 in PgAT, with increased numbers of CD11c<sup>+</sup> ATM (Fig. S2C-E). In addition, the lack of LXR $\beta$   
28 promoted a generalized reduction of ATM numbers, marked by a massive reduction in the  
29 numbers of CD206<sup>+</sup> ATM (Fig. S2F-H). Together, this data suggests that LXR $\alpha$  and LXR $\beta$  are  
30 important for preserve ATM numbers and have an overlapping function in the maintenance of  
31 CD11c<sup>+</sup> and CD206<sup>+</sup> ATM populations upon HFD, with distinct roles in CD11c<sup>+</sup> and CD206<sup>+</sup> ATMs  
32 when mice are fed a chow diet.

### 33 ***LXR $\beta$ deletion aggravates obesity-induced AT inflammation***

34 Since the concomitant deletion of LXR $\alpha$  and LXR $\beta$  did not change the CD11c/CD206 ratio in  
35 HFD-fed mice, we decided to investigate whether LXR may direct impact macrophage function,  
36 since ATMs numbers were reduced in both LXR $\alpha$  and LXR $\beta$  knockout animals. Because ATM  
37 reside in a lipid rich environment and lipid droplets are associated with a pro-inflammatory  
38 phenotype (22, 51), we evaluated the systemic effects of LXR deletion by analyzing the presence  
39 of lipid droplets in macrophages residing outside AT. LXR $\alpha$  $\beta$ KO macrophages isolated from the  
40 peritoneal cavity displayed elevated lipid droplet levels (Fig. 2E). Given that LXR deficient  
41 macrophages are loaded with lipid droplets, macrophages from WT, LXR $\alpha$  $\beta$ KO, LXR $\alpha$ KO and  
42 LXR $\beta$ KO mice were challenged with the TLR4 agonist LPS to evaluate their inflammatory fitness.  
43 Deletion of both LXR isoforms in macrophages resulted in increased LPS-induced IL-12 and  
44 TNF- $\alpha$  secretion (Fig. 2F). LXR $\beta$  and not LXR $\alpha$  mediated this enhanced effect on the secretion of  
45 IL-12 and TNF- $\alpha$  induced by LPS (Fig. 2F). Thus, LXR $\beta$  is required for macrophage inflammatory  
46 fitness and its absence potentiates macrophage pro-inflammatory profile.

47 Given that HFD consumption is associated with TLR4 activation in AT (52) and the role of LXR in  
48 the control of lipid homeostasis and regulation of immune responses (40), we next investigated  
49 whether the increased secretion of pro-inflammatory cytokines observed in LXR deficient  
50 macrophages could be extrapolated to ATM under metabolic stress induced by HFD. We  
51 observed that ATM from both HFD-fed LXR $\alpha$  $\beta$ KO and LXR $\beta$ KO mice have increased expression  
52 of IL-12, TNF- $\alpha$  and IL-1 $\beta$  compared with ATMs from WT mice fed with the same diet (Fig. 2G and  
53 Fig. S3). These data indicate that LXR $\beta$  is necessary to limit the secretion of pro-inflammatory  
54 cytokines by macrophages and that the deletion of LXR $\beta$  results in increased AT inflammation,  
55 which contributes to inflammation-induced insulin resistance and enhanced glucose intolerance  
56 (Fig. 1L-M).

1 **Expression of LXR in immune cells is necessary for fatty acid synthesis and body weight**  
2 **gain in HFD-fed animals**

3 Because we observed that LXR deficiency affects macrophage inflammatory fitness, we next  
4 hypothesized that the observed phenotype in whole body LXR deficient mice could be, at least in  
5 part, due to the lack of LXR specifically in immune cells, such as macrophages. Bone marrow  
6 cells from WT (WT→WT) or LXRαβKO (LXRαβKO→WT) animals were transferred into  
7 sublethally irradiated C57BL/6J WT recipient mice (Fig. 3A). These mice were then fed with HFD  
8 for 16 weeks. We observed that the lack of LXR in immune cells resulted in reduced HFD-  
9 induced weight gain (Fig. 3B) and reduced fat mass (Fig. 3C). No changes in food intake were  
10 observed between WT→WT and LXRαβKO→WT animals (Fig. 3D). LXRαβKO→WT animals had  
11 no changes in fasting blood glucose levels and glucose tolerance (Fig. 3E-F).

12 **LXR deficiency in immune cells increases the accumulation of pro-inflammatory ATMs in**  
13 **HFD-fed mice**

14 To investigate whether LXR deficiency in macrophages is involved in the increased AT  
15 inflammation observed in LXR deficient mice (Fig. 2), we evaluated ATM populations in PgAT of  
16 bone marrow transplanted animals (Fig. 3G-J). We observed that LXR have an essential role in  
17 the maintenance of AT immune homeostasis, since LXRαβKO→WT mice had increased AT  
18 inflammation, observed by increased number of ATMs in LXRαβKO→WT HFD-fed mice  
19 compared to WT→WT HFD-fed mice (Fig. 3G). Also, LXRαβKO→WT chow-fed mice had  
20 increased numbers of CD11c<sup>+</sup> ATMs compared to WT→WT chow-fed mice (Fig. 3G). The lack of  
21 LXR in immune cells increased the percentage of CD11c<sup>+</sup> in chow-fed mice, but not in HFD-fed  
22 mice (Fig. 3H). ATM isolated from PgAT of HFD-fed LXRαβKO→WT mice had increased  
23 expression of IL-1β and TNF-α (Fig. 3I and Extended Data Fig. 4A). Also, LXRαβKO→WT chow-  
24 fed mice displayed enhanced IL-1β and IL-12 expression in ATM of HFD-fed mice (Fig. 3I and  
25 Extended Data Fig. 4B). The increased monocyte infiltration in the PgAT on chow-fed mice (Fig.  
26 3J) indicate that LXR deficiency in immune cells imprints an immune imbalance that is  
27 exacerbated once mice is challenged with HFD. Together, these data indicate that LXR deficiency  
28 in immune cells increases obesity-induced AT inflammation through the enhancement of ATMs  
29 pro-inflammatory profile.

30 **Naringenin is an LXR agonist in macrophages and improves obesity-induced insulin**  
31 **resistance**

32 LXRα has been directly associated with increased fatty acid synthesis and administration of non-  
33 selective LXR agonists has been reported to increase blood triglyceride levels (41, 53-55). Thus,  
34 because of the detrimental effects of LXRα activation on lipid metabolism, the search for new  
35 selective LXRβ agonists has emerged as an attractive therapeutic target. Several compounds  
36 have been shown to modulate LXR, and, in some cases, display opposite effects in each LXR  
37 isoform (56). Among them, the citrus-derived flavonoid compound naringenin (NAR) has been  
38 shown to modulate LXR activity through controversial effects on LXRα (57, 58). For example,  
39 NAR treatment reduces fatty acid synthesis, alleviates atherosclerotic lesions (59) and promotes  
40 anti-inflammatory effects in mice (60, 61). The effects of NAR on LXRβ are poorly understood.  
41 Thus, we decided to investigate whether NAR could act as a modulator of LXR in macrophages  
42 and be used as a potential therapeutic approach to treat HFD-induced insulin resistance.  
43 Macrophages were treated with NAR and the expression of LXR target genes evaluated. We  
44 observed that similar to the widely used synthetic LXR agonist, GW3965, NAR-treated WT  
45 macrophages had increased expression of the LXR target genes *Abca1*, *Abcg1*, *ApoE* and *Lpl*,  
46 but not of the fatty acid synthesis-related gene *Fasn* (Fig. 4A), indicating that NAR acts as an  
47 LXR agonist in macrophages. NAR had no effect on LXRαβKO macrophages (Fig. S4C), which  
48 demonstrate that in macrophages NAR mediated effects are dependent on LXR expression. The  
49 absence of changes in *Fasn* expression raise the question if NAR could be binding preferentially  
50 in LXRβ in macrophages. Thus, a molecular docking analysis of NAR on LXRβ was carried out to  
51 probe the LXRβ-NAR interaction (Fig. S4D-E). The binding site of the lowest energy solution (-9.4  
52 kcal/mol; Fig. S4F) superposes relatively well to the interaction region of all 25 ligands (full and  
53 partial agonists and antagonists) which structures have been experimentally solved in complex  
54 with the LXRβ LBD. Thus, this indicates that the ligand binding domain of LXRβ can mediate the  
55 interaction with naringenin.  
56  
57

1 We next evaluated if NAR treatment modulates macrophage function. NAR treatment prior to  
2 LPS-induced macrophage activation resulted in reduced secretion of CCL2, IL-6 and TNF- $\alpha$ , and  
3 reduced expression of *Il-1 $\beta$*  (Fig. 4B). NAR treatment also increased the expression of the  
4 alternatively activated macrophage-related marker *Fizz1* (Fig. 4B), reinforcing the anti-  
5 inflammatory potential of NAR. TLR4 activation has been shown to impair cholesterol efflux in  
6 macrophages due to the inhibition of LXRs transcriptional activity (40). Also, the expression of  
7 *Abca1* has been proposed to play a critical role in the anti-inflammatory activity of LXRs (55).  
8 Consistent with these reports, we found that LPS-stimulated macrophages have reduced  
9 expression of *Abca1*, which was reversed by NAR treatment (Fig. 4B). Together, these data show  
10 that NAR is a potential LXR $\beta$  agonist with important anti-inflammatory properties.

### 11 ***Naringenin improves insulin sensitivity and adipose tissue inflammation in obese mice***

12 We next evaluated the *in vivo* effects of LXR activation with NAR in obesity and the subsequent  
13 effect on AT inflammation and insulin resistance. LXR activation was shown to have beneficial  
14 effects in obese and diabetic models (8). However, the clinical usage of LXR synthetic agonists  
15 are still a challenge because of elevated lipid accumulation and triglycerides levels (62). We  
16 observed that obese and insulin resistant mice treated with NAR for 4 weeks did not affect body  
17 weight, food intake and circulating levels of triglycerides (Fig. 4C-D). This indicate that NAR does  
18 not possess the harmful effects of other LXRs agonists on increasing the levels of triglycerides.  
19 Also, NAR-treated obese mice displayed improved insulin sensitivity compared to obese vehicle-  
20 treated group (Fig. 4E). Next, we investigated if the increased insulin sensitivity observed in NAR-  
21 treated animals could be associated with reduced AT inflammation. Obese mice displayed  
22 increased percentage and number of Pg ATMs (Fig. 4F). Treatment with NAR did not affect the  
23 total number of Pg ATM (Fig. 4F), but reduced the number of CD11c<sup>+</sup> ATM and monocyte in Pg  
24 AT of HFD-fed mice and increased the number of CD206<sup>+</sup> ATM in PgAT of chow-fed mice (Fig. 4F  
25 and Fig. S4G). Also, obese mice treated with NAR displayed reduced numbers of TNF- $\alpha$ <sup>+</sup> and  
26 IL-1 $\beta$ <sup>+</sup> ATMs in PgAT (Fig. 4F and Fig. S4H). Collectively, these data reinforce the therapeutic  
27 potential of NAR as an LXR agonist to decrease obesity-induced AT inflammation and ameliorate  
28 obesity-induced insulin resistance.  
29

### 30 ***Naringenin modulates the metabolism and function of macrophages through a LXR $\beta$ - 31 dependent mechanism***

32 Because we found that naringenin possess both *in vitro* and *in vivo* anti-inflammatory effects, we  
33 decided to further investigate if the mechanism through which NAR affects macrophages  
34 phenotype. LPS and IFN- $\gamma$  polarized macrophages displayed increased expression of pro-  
35 inflammatory markers, and increased expression of the glycolytic enzymes *Ldha* and *Pfkfb3*,  
36 which were all reduced upon NAR treatment (Fig. 4G). In addition, NAR treatment of non-  
37 polarized macrophages induced equivalent expression of *Nr1h3*, *ApoE* and *Abca1* to those found  
38 in IL-4-polarized macrophages (Fig. 4H). Moreover, NAR treatment also increased the expression  
39 of *Nr1h2* (LXR $\beta$ ) in IL-4-polarized macrophages (Fig. 4H). NAR treatment also reduced the  
40 expression of *Pfkfb3*, *Ldha* and *Nos2* in LPS-activated macrophages in a LXR $\beta$ -dependent  
41 manner (Fig. 4I). Because NAR reduced the expression of glycolytic enzymes in both LPS and  
42 IFN- $\gamma$  polarized- and LPS-activated macrophages (Fig. 4G-I), and that LPS activation induces the  
43 metabolic adaptation of macrophages marked by increased glycolysis and mitochondrial  
44 dysfunction (63-68), we hypothesized that the mechanism by which NAR modulates macrophage  
45 function may involve the metabolic adaptation of these cells. NAR treatment prior to LPS  
46 activation reduced LPS-induced glycolysis (Fig. 4J). Moreover, NAR treatment decreased the  
47 LPS-induced mitochondrial dysfunction in macrophages, indicating that NAR acts thorough LXR $\beta$   
48 to modulate macrophage metabolism and inflammatory fitness (Fig. 4J). Collectively, these data  
49 indicate that NAR-induced LXR $\beta$  activation limits macrophage activation, reduces glycolytic  
50 metabolism, and restores mitochondrial function.  
51  
52

### 53 **Discussion**

54 Obesity has reached epidemic rates worldwide and is a serious public health problem. Easy  
55 access to hyper caloric diets combined with technologic advances has promoted changes that  
56 favor a sedentary lifestyle and overweight. In combination with overweight, cardiovascular  
57 disorders, dyslipidemia and insulin resistance are frequently observed in obese subjects (69).  
58

1 Thus, the search for new therapeutic targets to treat obesity-associated disorders has become  
2 increasingly necessary.

3 Here, we describe a new role for LXR in the regulation of adipose tissue homeostasis. We show  
4 that LXR is a key factor for the maintenance of the immune balance in AT. Indeed, LXR effects  
5 has been extensively studied in the pathogenesis of atherosclerosis (70-72), and these studies  
6 have shed the light for LXR actions on macrophage metabolism and their influence in the whole  
7 body homeostasis. However, is poorly understood how LXR deficiency/activation may affect  
8 resident adipose tissue macrophages, specially during obesity. Moreover, few studies have  
9 focused in understand the roles of the distinct LXR isoforms (47). We decided to investigate how  
10 LXR $\alpha$  and LXR $\beta$  could modulate the whole body metabolism and macrophage response. We  
11 found that LXR isoforms display differential roles in vivo. Deletion of LXR $\beta$  and not of LXR $\alpha$   
12 promotes glucose intolerance. These data are consistent with previous works showing that LXR  
13 coordinates both lipid and glucose homeostasis (1, 10, 73). LXR has been showed to promote the  
14 transcriptional induction of Glut4 (10), we extrapolate that LXR $\beta$  may exert an essential role in  
15 this process, however, further analysis must be performed in order to verify such function for this  
16 LXR isoform.

17 Together with the changes in adipocyte size and adipokine secretion, obesity triggers changes in  
18 the AT resident immune population (26, 32, 33, 36, 38), generating an inflammatory  
19 microenvironment marked by the presence of CD11c+ macrophages secreting pro-inflammatory  
20 cytokines and enhanced monocyte infiltration (35, 74, 75). Our data showed that the absence of  
21 LXR *per se* is sufficient to lead the adipose tissue for an obesity-like inflamed status, in which the  
22 imbalance between CD11c+ and CD206+ macrophages is observed. Moreover we found that  
23 LXR $\beta$  deletion aggravates HFD-induced AT inflammation and LXR deletion specifically in immune  
24 cells leads to AT inflammation, observed by increased pro-inflammatory ATM accumulation.  
25 Pro-inflammatory cells presents a unique metabolic signature, which is characterized by increased  
26 glycolytic pathway activity, the disruption of tricarboxylic acid (TCA) cycle and mitochondrial  
27 dysfunction (63, 76-78). We found that naringenin is an LXR agonist which modulates  
28 macrophage metabolism and function by reducing glycolysis, mitochondrial dysfunction and the  
29 subsequent pro-inflammatory phenotype.

30 Other groups has demonstrate that the expression of the LXR target gene *Abca1* is essential for  
31 macrophage reverse cholesterol transport, and its absence is causally linked to the pathogenesis  
32 of atherosclerosis (72). Our findings reveal that the pro-inflammatory stimulus impairs *Abca1*  
33 expression (Fig. 4B) and increase lipid droplet accumulation in macrophages (data not shown),  
34 driving the macrophages to a pro-inflammatory profile, and NAR induces *Abca1* in an LXR  
35 dependent manner.

36 A variety of studies aimed to understand how LXR modulates the immune system and how the  
37 anti-inflammatory effects promoted by the LXR activation could be used to treat immune-  
38 mediated diseases (42, 43, 55, 79-82). However, classical LXR agonists has been showed to  
39 induce the expression of genes related to fatty acid synthesis, and thus, promoting increased  
40 levels of triglycerides in the blood and liver (83, 84), specially by the activation of LXR $\alpha$ . Finally,  
41 we described that naringenin is a LXR $\beta$  agonist in macrophages, and naringenin-induced LXR $\beta$   
42 activation improves obesity-induced insulin resistance and reduces AT inflammation, with no  
43 effects on triglycerides synthesis. However, we cannot exclude the fact that LXR acts in several  
44 other cell types, such as hepatocytes, adipocytes, neurons, beta cells, among others, to regulate  
45 whole body homeostasis, which could only be promptly evaluated in future works with access to  
46 LXR-floxed mice. Our work focuses on understanding how specific isoforms of liver X receptors  
47 from adipose tissue macrophages can modulate adipose tissue inflammation and insulin  
48 sensitivity. Collectively, our data reinforce that LXR $\alpha$  and LXR $\beta$  display distinct roles in the  
49 regulation of macrophage response and whole-body glucose homeostasis. This study contributes  
50 for a better understand on how nuclear receptors isoforms may display distinct functions and  
51 serves as the first step for the development of new therapies targeting LXR $\beta$  in the treatment of  
52 immune-mediated metabolic diseases.

## 53 **Materials and Methods**

54 A detailed methods employed in this paper is available in SI appendix, and include the following  
55 topics:

56 Animal studies and metabolic analysis  
57

- 1 - Ethics and genetic background
- 2 - Diet-induced obesity
- 3 - In vivo naringenin treatment
- 4 - Glucose and Insulin Tolerance Test
- 5 - Triglycerides
- 6 - Evaluation of energy metabolism
- 7 - Evaluation of adipose tissue inflammation
- 8 - Bone marrow transplant

#### 9 In vitro macrophage analysis

- 10 - Cell culture
- 11 - Cytokine secretion evaluation
- 12 - Lipid droplet staining
- 13 - RNA extraction and cDNA synthesis
- 14 - Gene expression analysis by RT-qPCR
- 15 - Macrophage metabolism assay
- 16 - Mitochondrial potential evaluation

17 Analysis from RNA sequencing data.

18 Docking

19 Statistical analysis

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25

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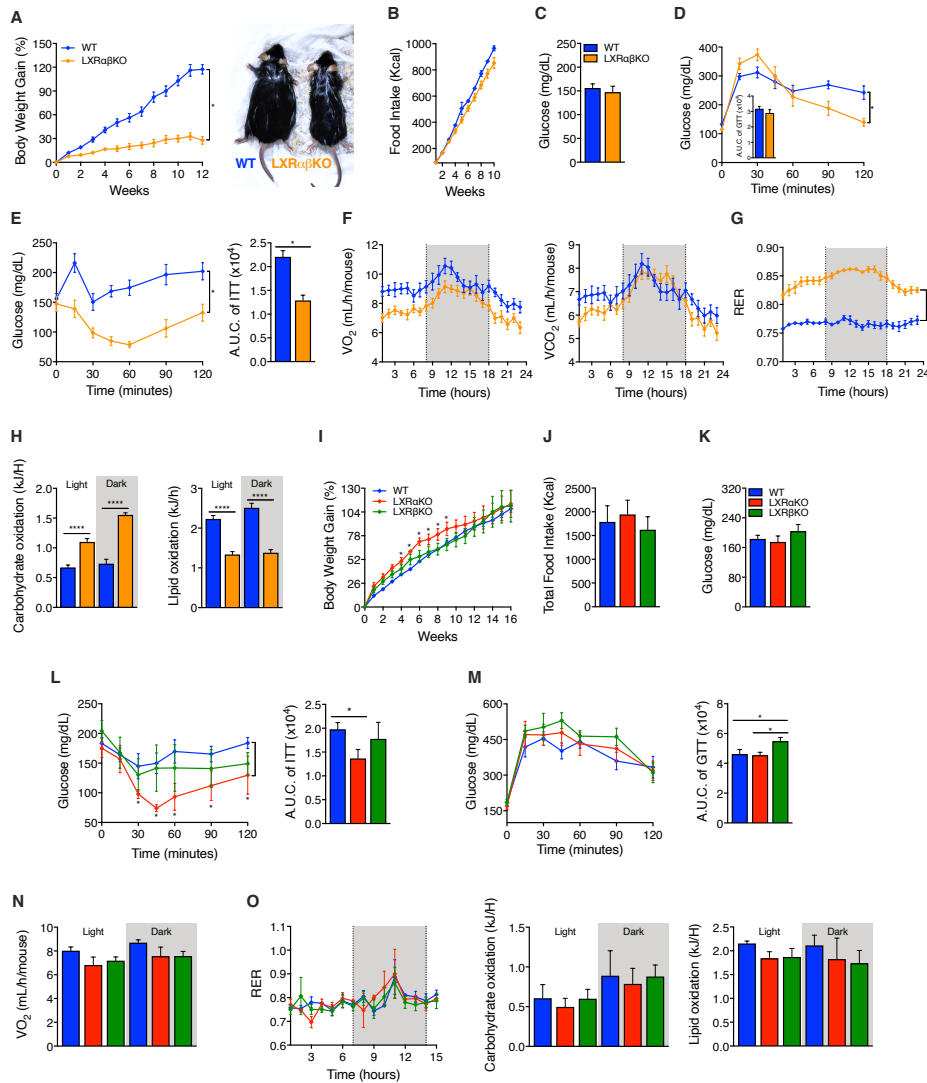


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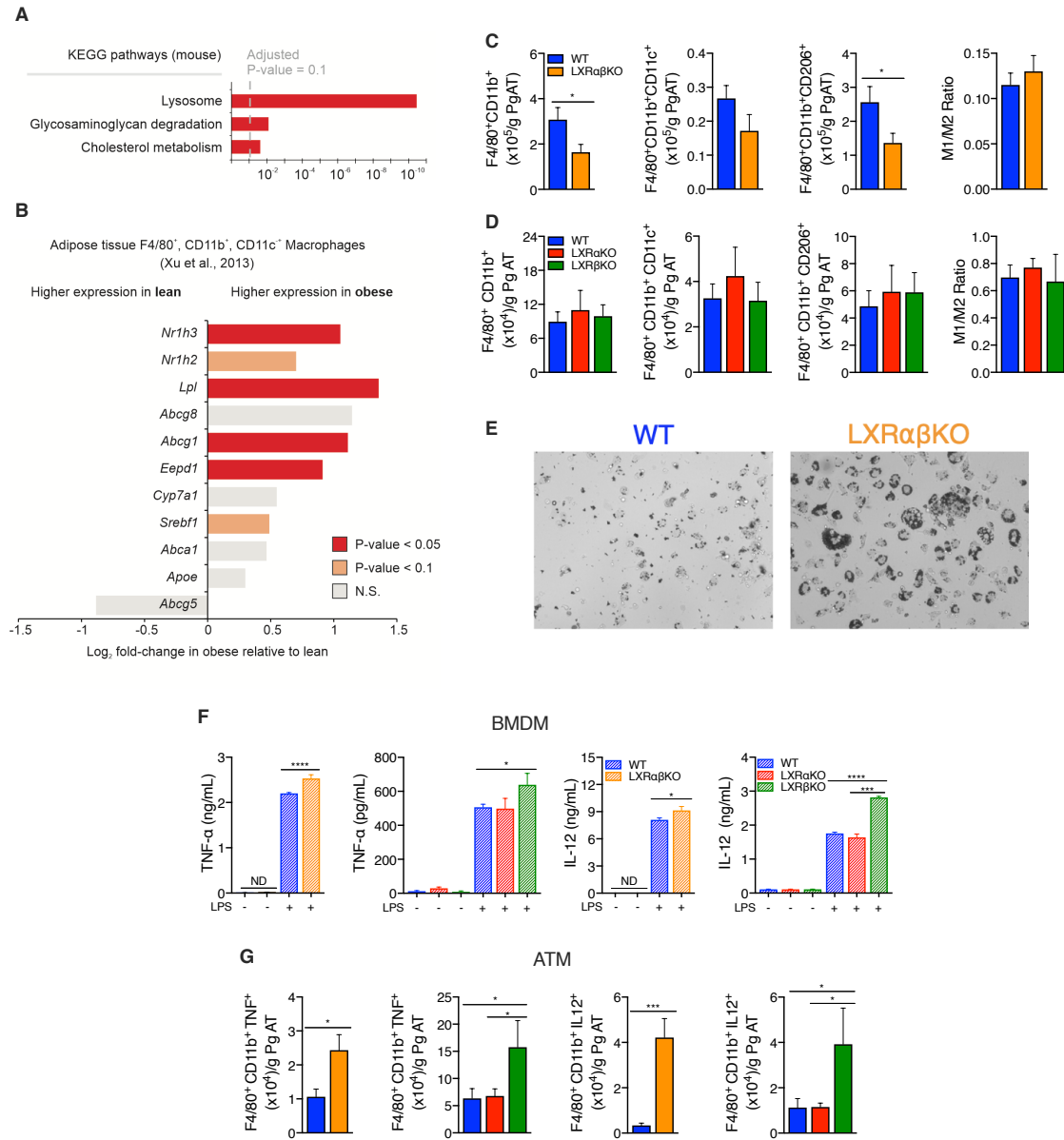
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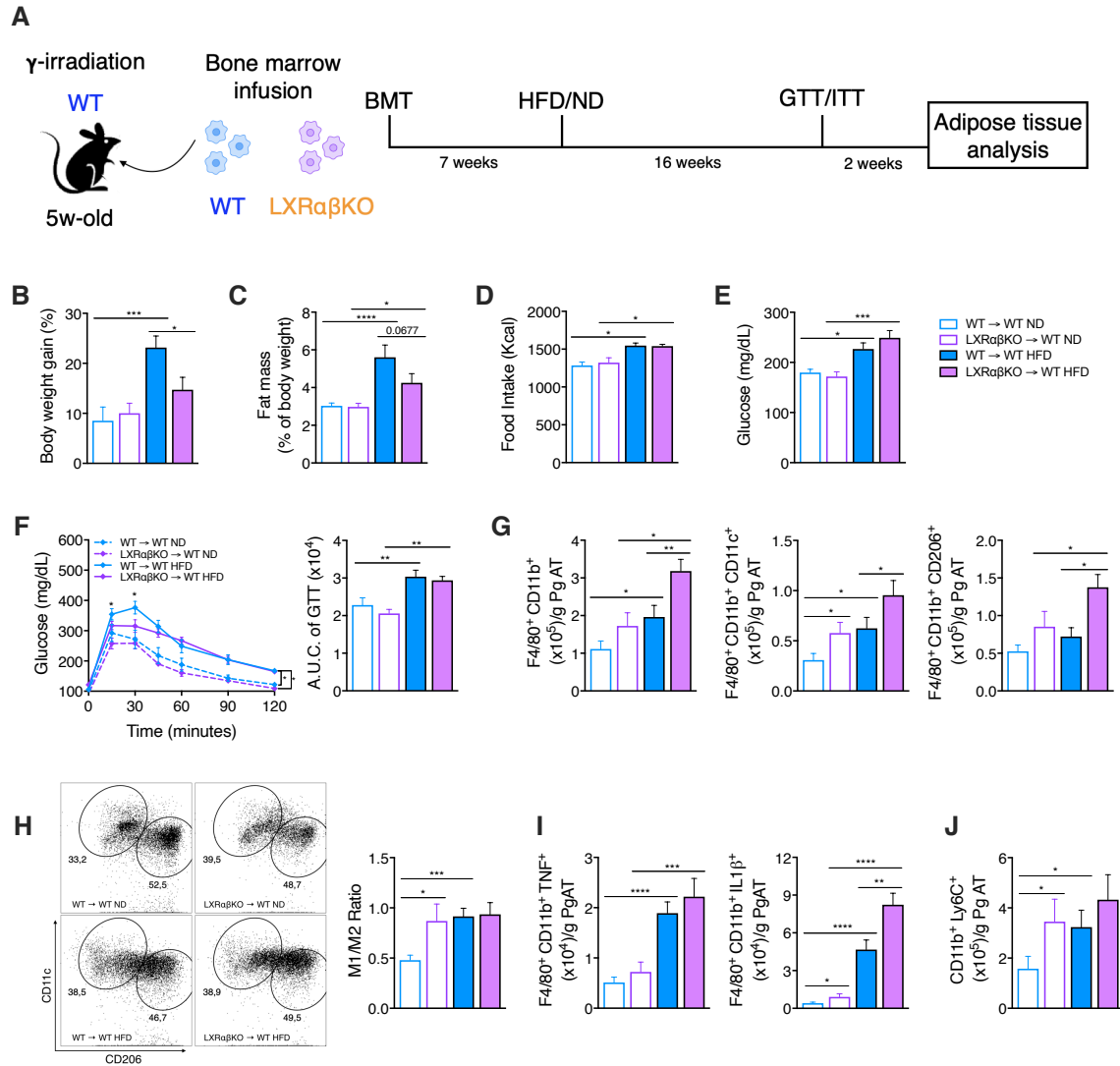
## Figures and Tables



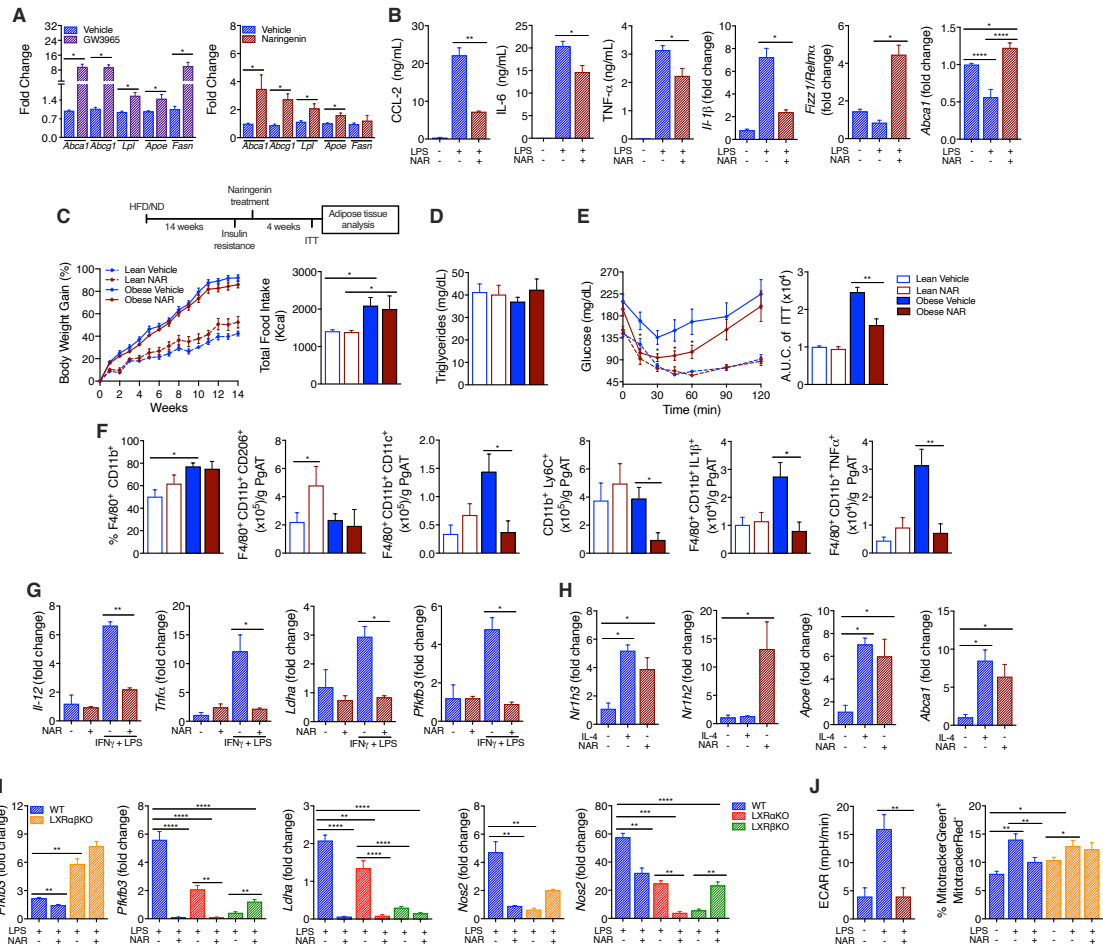
**Fig. 1. LXR $\alpha$  and LXR $\beta$  display distinct role in the metabolic homeostasis.** (A) Body weight gain and representative image of WT and LXR $\alpha\beta$ KO HFD-fed mice (n= 7 mice/group). (B) Food intake in HFD fed animals (n= 7 mice/group). (C) Blood glucose levels after 5 hours of fasting (n= 7 mice/group). (D) Glucose Tolerance Test (GTT) and area under curve (AUC) (n= 7 mice/group). (E) Insulin Tolerance Test (ITT) and area under curve (AUC) (n= 7 mice/group). (F) Wild type and LXR $\alpha\beta$ KO mice were housed under 12 h light-dark cycles, and metabolic cages were used to monitor oxygen consumption rate (VO<sub>2</sub>) and carbon dioxide consumption rate (VCO<sub>2</sub>) (n= 7 mice/group). (G) The respiratory exchange ratio (RER) was calculated by the ratio between VCO<sub>2</sub> and VO<sub>2</sub> (n= 7 mice/group). (H) Carbohydrate and fat oxidation in WT and LXR $\alpha\beta$ KO mice was calculated using VO<sub>2</sub> and VCO<sub>2</sub> values (n= 7 mice/group). (I) Body weight gain in WT, LXR $\alpha$ KO and LXR $\beta$ KO mice HFD-fed mice (n = 6 mice/group). (J) Total food intake in WT, LXR $\alpha$ KO and LXR $\beta$ KO mice HFD-fed mice (n = 6 mice/group). (K) Blood glucose levels in WT, LXR $\alpha$ KO and LXR $\beta$ KO HFD-fed mice after 5 hours of fasting (n = 6 mice/group). (L) ITT and AUC of HFD WT, LXR $\alpha$ KO and LXR $\beta$ KO mice (n = 6 mice/group). (M) Glucose Tolerance Test (GTT) in WT, LXR $\alpha$ KO and LXR $\beta$ KO HFD-fed mice (n = 6 mice/group). (N) O<sub>2</sub> consumption (VO<sub>2</sub>) in WT, LXR $\alpha$ KO and LXR $\beta$ KO HFD-fed mice, evaluated in metabolic cage (n = 6 mice/group). (O) Evaluation of RER, carbohydrate oxidation and lipid oxidation in WT, LXR $\alpha$ KO and LXR $\beta$ KO HFD-fed mice, evaluated in metabolic cage (n = 6 mice/group). Values in the graph represents mean  $\pm$  SEM. (A-J) t test, \*p<0.05, \*\*p<0.01, and \*\*\*p<0.001. (K-O) One-way ANOVA, *post-hoc* Bonferroni test, \*p<0.05.



**Fig. 2. The lack of LXR impairs the immune balance in PgAT. (A)** KEGG pathway enrichment analysis in adipose tissue macrophages from lean and obese WT mice. **(B)** Transcriptomic profile of ATM (F4/80<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>-</sup>) in lean and obese WT animals. Values in the graph are represented as the following: grey  $p > 0.1$ ; orange  $p < 0.1$  and red  $p < 0.05$ . **(C)** Adipose tissue macrophage population of WT and LXR $\alpha\beta$ KO in PgAT of HFD-fed animals ( $n=7$  mice/group). **(D)** Macrophage population in PgAT of HFD WT, LXR $\alpha$ KO and LXR $\beta$ KO animals ( $n=6$  mice/group). **(E)** Lipid droplet evaluation in WT and LXR $\alpha\beta$ KO peritoneal macrophages stained with oil red O. **(F)** Evaluation of IL-12 and TNF- $\alpha$  secretion in WT and LXR deficient BMDMs after 24 hours of LPS stimuli. **(G)** IL-12 and TNF- $\alpha$  expression in ATM from WT, LXR $\alpha$ KO and LXR $\beta$ KO HFD-fed mice ( $n=6$  mice/group) and WT and LXR $\alpha\beta$ KO HFD-fed mice ( $n=7$  mice/group). Values in the graph represents mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .



**Fig. 3. LXR deletion in macrophages induces adipose tissue inflammation in lean animals.** (A) Illustration showing the bone marrow transplantation (BMT) protocol in chow- and HFD-fed mice. (B) Body weight gain in response to BMT in WT→WT and LXRαβKO→WT chow- and HFD-fed mice (n=10 mice/group). (C) White adipose tissue mass in WT→WT and LXRαβKO→WT chow- and HFD-fed mice (n=10 mice/group). (D) Total food intake in BMT chow- and HFD-fed mice (n=10 mice/group). (E) Fasting blood glucose levels in WT→WT and LXRαβKO→WT chow- and HFD-fed mice (n=10 mice/group). (F) Glucose tolerance test (n=10 mice/group). (G) Adipose tissue macrophage populations in PgAT of BMT animals (n=10 mice/group). (H) Immune balance between M1 and M2 ATM (n=10 mice/group). (I) TNF-α and IL-1β expression in ATM from BMT mice (n=10 mice/group). (J) Monocyte infiltration in PgAT of WT→WT and LXRαβKO→WT chow- and HFD-fed mice (n=10 mice/group). Values in the graph represents mean ± SEM.  $p \leq 0.05$ ,  $p^{***} \leq 0.005$ ,  $p^{****} \leq 0.0005$ ,  $p^{*****} \leq 0.0001$ .



**Fig. 4. LXR $\beta$  activation improves insulin sensitivity by reduce adipose tissue inflammation in obese animals. (A)** LXR target genes expression in WT macrophages treated with GW3965 or Naringenin (NAR). **(B)** Evaluation of NAR treatment over the response of LPS-activated macrophages. **(C)** Illustration showing in vivo LXR activation protocol (upper), Body weight gain and food intake. **(D)** Blood triglycerides levels were determined by colorimetric assay after 5 hours of fasting. **(E)** Insulin tolerance test in control and NAR-treated animals. **(F)** Evaluation of PgAT inflammation in response to LXR activation. **(G)** Effects of LXR activation in macrophages polarized with LPS+IFN $\gamma$ . **(H)** LXR and LXR target genes expression in IL-4-stimulated macrophages. **(I)** Pro-inflammatory markers expression in LPS-activated WT and LXR deficient macrophages. **(J)** Glycolytic metabolism measured by Seahorse and mitochondrial dysfunction evaluation by flow cytometry in WT and LXR deficient macrophages in response to NAR treatment. Values in the graph of in vivo experiments represents mean of 8 animals/group  $\pm$  SEM. In vitro experiments graphs are the representative for three independent experiments.  $p \leq 0.05$ ,  $p^{***} \leq 0.005$ ,  $p^{****} \leq 0.0005$ ,  $p^{*****} \leq 0.0001$ .

## **APENDICE 5**





# Self-tunable engineered yeast probiotics for the treatment of inflammatory bowel disease

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**Inflammatory bowel disease (IBD) is a complex chronic inflammatory disorder of the gastrointestinal tract. Extracellular adenosine triphosphate (eATP) produced by the commensal microbiota and host cells activates purinergic signaling, promoting intestinal inflammation and pathology. Based on the role of eATP in intestinal inflammation, we developed yeast-based engineered probiotics that express a human P2Y2 purinergic receptor with up to a 1,000-fold increase in eATP sensitivity. We linked the activation of this engineered P2Y2 receptor to the secretion of the ATP-degrading enzyme apyrase, thus creating engineered yeast probiotics capable of sensing a pro-inflammatory molecule and generating a proportional self-regulated response aimed at its neutralization. These self-tunable yeast probiotics suppressed intestinal inflammation in mouse models of IBD, reducing intestinal fibrosis and dysbiosis with an efficacy similar to or higher than that of standard-of-care therapies usually associated with notable adverse events. By combining directed evolution and synthetic gene circuits, we developed a unique self-modulatory platform for the treatment of IBD and potentially other inflammation-driven pathologies.**

IBD is a complex chronic inflammatory disorder of the gastrointestinal tract that includes Crohn's disease and ulcerative colitis<sup>1</sup>. Most available IBD therapies suppress the immune system systemically, increasing the risk of infections and some types of cancer while not benefiting all patients<sup>2</sup>. Hence, there is an unmet clinical need for localized and tunable IBD therapies.

The microbiome controls important immune functions in health and also in the context of multiple diseases including IBD<sup>3–5</sup>. The role of the microbiome in disease pathogenesis and anti-inflammatory effects of certain commensal microorganisms support the use of probiotic-based therapies for IBD<sup>6</sup>. Unmanipulated probiotics, however, use built-in anti-inflammatory mechanisms, which have been evolutionarily selected to optimize host–commensal interactions in the healthy gut but not in the context of pathologic inflammation<sup>7</sup>.

Recent advances in synthetic biology enabled engineering of probiotics to deliver therapeutic proteins in response to disease-associated signals<sup>8–10</sup>. One such signal relevant to IBD is eATP, which is released by activated immune cells and commensal bacteria. eATP signals via purinergic receptors to boost pro-inflammatory cytokine production and effector T cell activation, suppress regulatory T cell ( $T_{reg}$ ) responses and promote enteric neuron apoptosis, among other biological responses thought to contribute to IBD pathology<sup>11–14</sup>. eATP signaling is limited by the membrane-bound ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1 or CD39), which hydrolyzes eATP into AMP; CD73 then metabolizes AMP into immunosuppressive adenosine. CD39 limits eATP-driven pro-inflammatory responses, while it boosts

differentiation, stability and function of  $T_{reg}$  cells<sup>14–16</sup>. Purinergic signaling is therefore a candidate therapeutic target for IBD, with the caveat that, although adenosine has acute anti-inflammatory effects, persistent activation of adenosine signaling can cause fibrosis, which is further exacerbated by cytokines<sup>17,18</sup>. Thus, therapeutic targeting of purinergic signaling requires a regulated approach to intervene when and where needed, responsive to pro-inflammatory concentrations of eATP but self-limited to minimize the side effects associated with persistent adenosine signaling.

*Saccharomyces* yeast species have long been known for their use in foods, and certain species are considered safe probiotics<sup>19</sup>. *Saccharomyces cerevisiae* has a well-defined signal transduction pathway, the mating pathway, that can be functionally linked to human G protein-coupled receptors (GPCRs), enabling controlled expression of proteins in response to stimuli relevant to human physiology<sup>20,21</sup>. We combined directed evolution and synthetic biology approaches to develop *S. cerevisiae* probiotics, which, in response to eATP detected via an engineered human P2Y2 GPCR, secrete the CD39-like eATP-degrading enzyme apyrase. These self-tunable engineered probiotic yeasts suppress experimental intestinal inflammation in mice and could provide a new therapeutic platform for IBD and other inflammatory disorders.

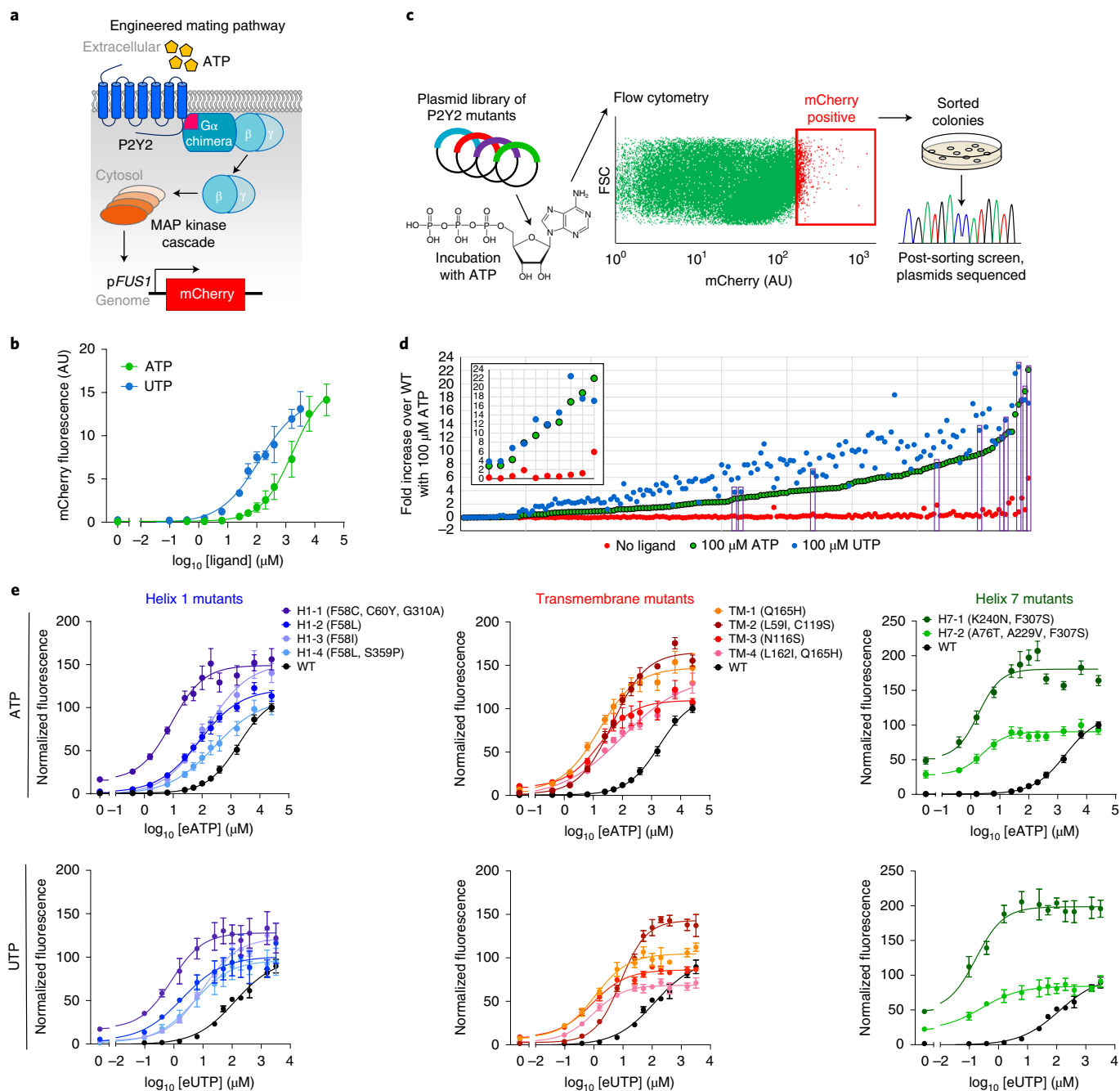
## Results

**Directed evolution of the human P2Y2 receptor.** The P2Y2 receptor is a GPCR that senses eATP and also extracellular uridine triphosphate (eUTP)<sup>17</sup> and has previously been functionally expressed

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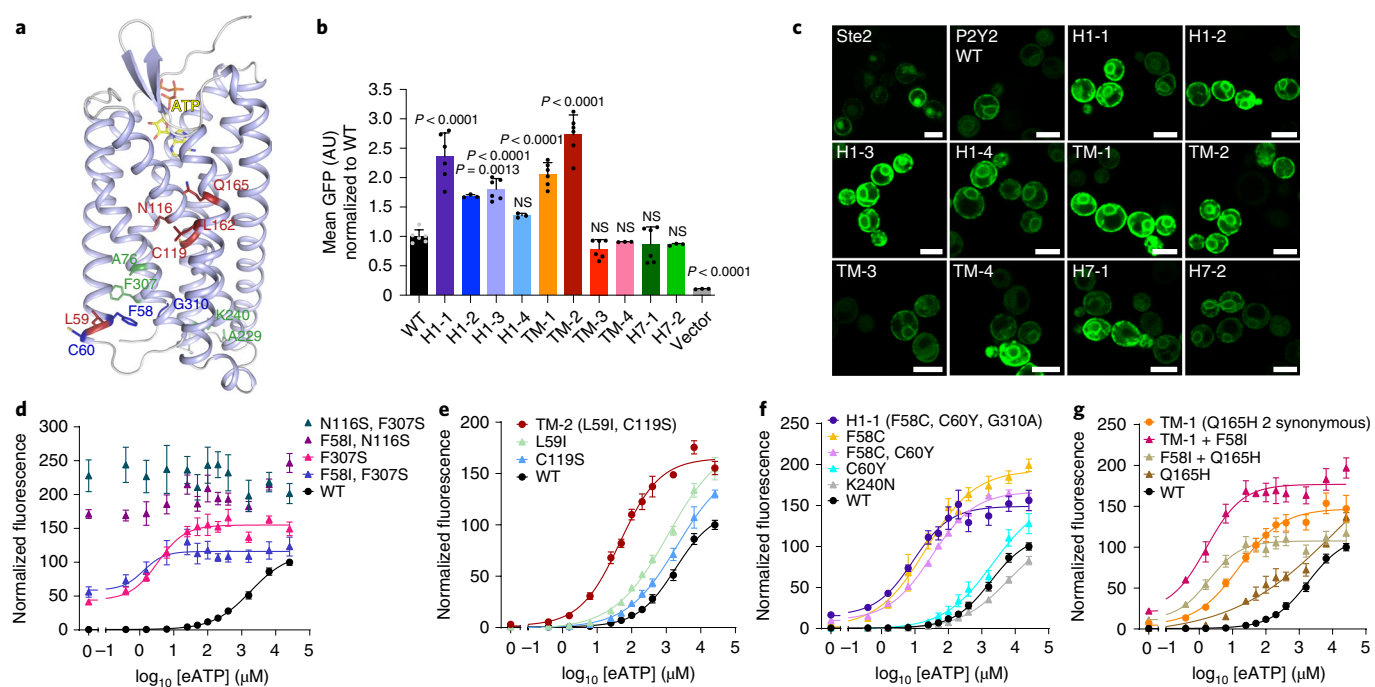
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**Fig. 1 | Directed evolution of the human P2Y2 receptor.** **a**, Schematic of engineered human P2Y2 receptor activation functionally coupled to the expression of a fluorescent reporter protein, mCherry, using the mating-responsive promoter *pFUS1*. Modifications to the mating pathway included knockout of genes encoding the negative regulator *Sst2* and *Far1*, which halts cell growth in the WT mating pathway. The chimeric G $\alpha$  protein (Gpa1-G $\alpha_3$ ) contains the five C-terminal amino acids of mammalian G $\alpha_3$ . MAP, mitogen-activated protein. **b**, Cells expressing the human WT P2Y2 receptor were treated with UTP or ATP, and mCherry fluorescence was quantified by flow cytometry.  $n=6$  colonies for ATP,  $n=3$  colonies for UTP. Data points represent the mean, and error bars represent s.e.m. AU, arbitrary units; [ligand], ligand concentration. **c**, A plasmid library of mutants for the human P2Y2 receptor generated by error-prone PCR was transformed into the Gpa1-G $\alpha_3$  mCherry reporter strain. Cells were treated with 100  $\mu$ M ATP, and flow cytometry was used to select for highly activating mutants (top 1% of mCherry fluorescence). Individual yeast colonies were then screened to confirm the desired phenotype and sequenced. FSC, forward scatter. **d**, Randomly selected yeast colonies were incubated with the indicated ligand for 6 h, and mCherry fluorescence was quantified. Data represent fold increase over the response from WT human P2Y2 receptor activated with 100  $\mu$ M ATP. Ten yeast colonies were selected for detailed characterization (purple boxes); their responses to 100  $\mu$ M ATP and 100  $\mu$ M UTP are shown in the inset. **e**, Many mutations in the human P2Y2 receptor increased the sensitivity and maximum response to eATP and eUTP. mCherry fluorescence is represented as a percentage of the maximum WT response to eATP. Mutants are grouped based on the location of mutant residues.  $n=6$  colonies for the response to eATP and  $n=3$  colonies for the response to eUTP. Data points represent the mean, and error bars represent s.e.m.

in yeast<sup>20</sup>. Both eUTP and eATP are released from mammalian cells, but, due to lower intracellular concentrations of UTP, released eUTP concentrations are only 10–30% those of eATP<sup>22</sup>. Importantly,

eATP, but not eUTP, activates P2X7 receptor signaling<sup>17</sup>, which is associated with intestinal pathology<sup>13,23</sup>. We first engineered the human P2Y2 receptor to increase its sensitivity to eATP when



**Fig. 2 | Characterization of human P2Y2 receptor mutants.** **a**, Human P2Y2 receptor structure with residues mutated following directed evolution highlighted. The top ten mutant P2Y2 receptors with enhanced ATP sensitivity were grouped based on the location of key mutant residues: helix 1 (blue), helix 7 (green) and the transmembrane region (red). **b**, Expression of C-terminal GFP-tagged human P2Y2 receptor mutants in yeast was quantified by flow cytometry. Mean GFP values were normalized to that of WT P2Y2-GFP.  $n=3$  colonies for H1-2, H1-4, TM-4, H7-2 and vector and  $n=6$  for the rest; error bars represent s.d. One-way ANOVA, Dunnett's post hoc test; NS, not significant;  $P$  values are indicated versus WT P2Y2-GFP. **c**, GFP-tagged endogenous yeast Ste2 GPCR and human P2Y2 receptor mutants were examined by confocal microscopy. Scale bars, 5  $\mu\text{m}$ ; images are representative of two independent experiments. **d–g**, eATP sensitivity of mutant mCherry fluorescence is represented as a percentage of the maximum WT response to eATP. Data points represent the mean. **d**, The combination of the N116S substitution with either F58I or F307S resulted in fully constitutively active human P2Y2 receptor. The F307S substitution alone conferred constitutive activity, improved sensitivity and improved maximum response to eATP.  $n=6$  colonies per group. **e**, Non-additive effects contributed to increased activity of the human P2Y2 receptor TM-2 mutants. The L59I and C119S substitutions alone conferred a moderate increase in sensitivity to eATP. The combined effect was less than the one detected in the TM-2 mutant, indicating a non-additive change.  $n=6$  colonies per group. **f**, By analyzing each mutation in the H1-1 mutant separately, F58C was identified as the primary substitution influencing activity. The K240N substitution from the H7-1 mutant did not increase eATP sensitivity.  $n=6$  colonies for H1-1 and WT,  $n=3$  otherwise. **g**, The TM-1 mutant harbored synonymous mutations, in addition to the Q165H substitution. The synonymous mutations contributed to increased eATP sensitivity, including when they were combined with the F58I substitution.  $n=6$  colonies for H1-1 and WT,  $n=3$  otherwise. Error bars represent s.e.m.

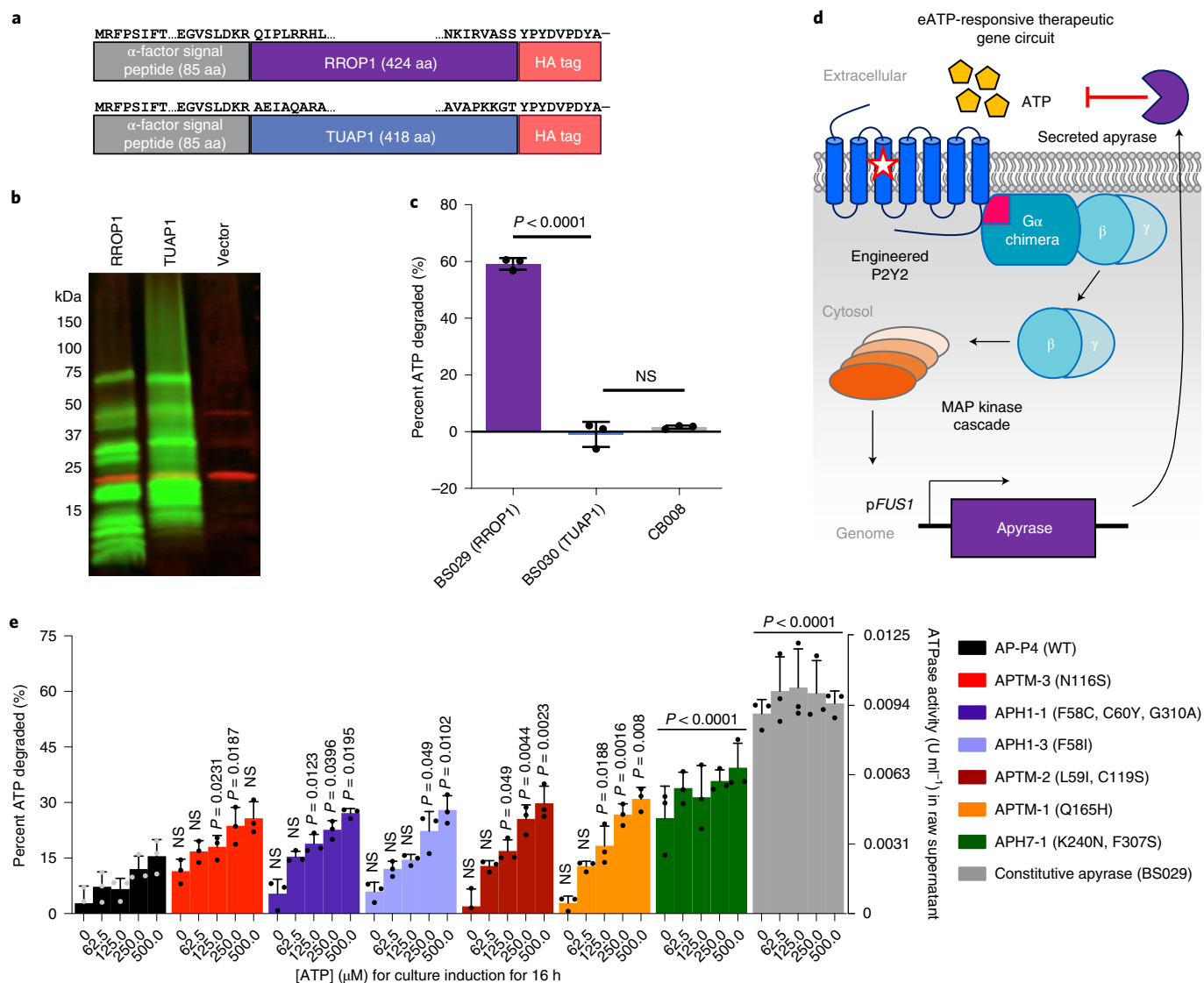
expressed in yeast. To establish a platform amenable to directed evolution, we coupled the human P2Y2 receptor to the yeast mating pathway via a chimeric yeast Gpa1–human  $G\alpha_{13}$  protein and monitored pathway activation using a fluorescent mCherry reporter controlled by the mating-responsive *FUS1* promoter (*pFUS1*) (Fig. 1a, Supplementary Table 1 and Extended Data Fig. 1a–c) (Reporter yeast strains in the Methods). The Gpa1– $G\alpha_{13}$  *pFUS1*-mCherry strain transformed with a plasmid constitutively expressing human P2Y2 showed a dose-dependent response to its agonists eATP and eUTP, with log half-maximum effective concentrations ( $\log EC_{50}$ ) of 3.27 and 2.09  $\mu\text{M}$ , respectively (Fig. 1b) (Flow cytometry evaluation of response to ATP and UTP in the Methods).

Physiological eATP levels associated with inflammation have been detected in the 100  $\mu\text{M}$  to high mM range<sup>34</sup>. However, yeast expressing wild-type (WT) P2Y2 showed a weak response to 100  $\mu\text{M}$  eATP, as determined by flow cytometry analysis of mCherry expression (Fig. 1b). Thus, we applied directed evolution to generate yeast-expressed human P2Y2 receptor mutant proteins that show increased sensitivity to eATP. To achieve this goal, we generated a plasmid library of human P2Y2 receptor mutants using error-prone PCR (Supplementary Table 2) and then isolated yeast by flow cytometry expressing the highest (top 1%) levels of *pFUS1*-driven mCherry fluorescence after treatment with 100  $\mu\text{M}$  eATP (Fig. 1c) (Directed evolution of human P2Y2 receptor in the Methods).

We performed multiple iterative rounds of flow cytometry-based selection to isolate mutants displaying the desired increase in eATP sensitivity (Extended Data Fig. 2). Finally, in a post-sorting screening step, we further assessed the function of the engineered human P2Y2 receptor mutants (Fig. 1d). For most of the analyzed colonies, an increased response to eATP was concomitant with an increased response to eUTP.

We focused on human P2Y2 receptor mutants that showed an enhanced response to eATP and a high eATP/eUTP response ratio concomitant with no constitutive expression of mCherry. Plasmid sequencing revealed a diverse range of P2Y2 genotypes (gene symbol *P2RY2*), with up to three nonsynonymous mutations in the gene encoding the receptor (Supplementary Table 3). Eight of the 19 human P2Y2 mutants harbored a mutation at site F58<sup>1,57</sup>, and we also observed mutations at nearby residues L59<sup>1,58</sup> and C60<sup>1,59</sup> and at Q165<sup>4,57</sup> and F307<sup>7,54</sup>.

We selected ten P2Y2 mutants for detailed characterization; each mutant was named using a unique identifier based on the location of the mutated residue(s) (Supplementary Table 4). In dose–response studies, the engineered P2Y2 receptors were more sensitive to both eATP and eUTP (Fig. 1e). When compared to the WT human P2Y2 receptor, the selected mutants showed a 10–1,000-fold decrease in the  $EC_{50}$  for eATP and up to a 1.8-fold increase in the maximum mating pathway response. This increased sensitivity was also observed



**Fig. 3 | eATP-responsive secretion of ATPase by engineered yeast.** **a**, Modified apyrase peptide sequences used as therapeutic response elements. Natural signal peptides were replaced with the yeast  $\alpha$ -factor signal peptide to facilitate secretion, and an HA tag was added to the C terminus. aa, amino acids. **b**, Cell lysates from yeast strains constitutively expressing potato apyrase (RROP1) or wheat apyrase (TUAP1) or not expressing any apyrase (vector). Green bands correspond to the HA tag on apyrase; red bands indicate the cytoplasmic protein Pgk1 loading control. Image is representative of two independent experiments. **c**, Culture supernatant from strains constitutively secreting potato apyrase (RROP1) or wheat apyrase (TUAP1) were incubated for 30 min with ATP at 50  $\mu\text{M}$ , and residual ATP levels were quantified; the yeast parent strain that does not express apyrase was used as a negative control (CB008).  $n = 3$  samples per group. One-way ANOVA, Tukey's post hoc test. **d**, Schematic of engineered human P2Y2 receptor activation functionally coupled to the expression of RROP1 apyrase using the mating-responsive promoter *pFUS1*. Upon activation of the receptor by eATP, apyrase is expressed and secreted by the yeast. Secreted apyrase dephosphorylates eATP, in turn shutting off the gene circuit. **e**, Engineered yeast strains harboring a P2Y2-RROP1 gene circuit were incubated for 16 h with the indicated concentration of ATP. ATPase activity was quantified by incubating culture supernatant with 50  $\mu\text{M}$  ATP for 30 min and then measuring residual ATP levels. ATPase unit = 1  $\mu\text{mol}$  ATP to ADP per min.  $n = 3$  samples per group. Two-way ANOVA, Dunnett's post hoc test, versus the WT response at the same ATP concentration.  $P$  values are indicated. Error bars represent s.d.

in response to eUTP stimulation, except for strains harboring the Q165H<sup>4,57</sup> substitution (TM-1, TM-4), in which the maximum mating pathway response was 1.4-fold to twofold higher for eATP than that for eUTP. Thus, the application of directed evolution resulted in the generation of human P2Y2 receptor mutants with increased responsiveness to eATP.

**P2Y2 receptor mutants with increased sensitivity to eATP.** Key residues that participate in the binding of nucleotides and the activation of human P2Y2 receptor have been identified<sup>25</sup>, but mutations detected in the selected P2Y2 receptor mutants did not involve

these residues. Instead, the mutations that we identified involved residues peripheral to the ligand-binding pocket or residues located on the intracellular-facing side of the receptor (Fig. 2a).

To determine the molecular mechanisms responsible for the increased sensitivity of the selected P2Y2 receptor mutants, we analyzed their expression levels using receptor mutants tagged with C-terminal GFP. Expression in yeast was increased when F58<sup>1,57</sup> was mutated to a smaller hydrophobic residue ('H1' mutants) and also in transmembrane (TM)-1 and TM-2 mutants (Fig. 2b,c and Supplementary Table 3). However, these mutations did not involve residues homologous to previously described mutations that

improve human GPCR expression in yeast<sup>26</sup>. Thus, our findings identify unique residues that influence human P2Y2 expression.

We detected increased responsiveness and signaling in the absence of agonist (constitutive activity) in P2Y2 mutants harboring the N116S<sup>3,35</sup> and F307S<sup>7,54</sup> substitutions (TM-3, H7-1 and H7-2 mutants) (Fig. 1e). Interestingly, these mutants showed expression levels similar to those of the WT P2Y2 receptor. The N116S<sup>3,35</sup> substitution likely disrupts a conserved hydrogen-bonding network with D79<sup>2,50</sup> and N298<sup>7,45</sup>, and the lack of these stabilizing inter-helical interactions leads to increased signaling in the absence of agonist<sup>27</sup>. Based on known interactions of similar residues in other GPCRs, we predict that the F307S<sup>7,54</sup> substitution facilitates rotation of Y<sup>7,53</sup> into the active conformation, resulting in constitutive activity and increased eATP sensitivity<sup>28</sup>.

To further investigate mechanisms responsible for the differential activity of P2Y2 receptor mutants, we evaluated the effects of mutations alone or in combination. Certain substitutions (C60Y, K240N, G310A) did not cooperate to further increase P2Y2 receptor responsiveness to eATP, while others had deleterious effects (A76T and A229V, L162I, S359P) or exhibited positive epistasis when combined (N116S with F58I or F307S, L59I and C119S) (Fig. 2d–f). Synonymous mutations in the TM-1 mutant sequence increased P2Y2 receptor sensitivity toward eATP, which could be further increased by incorporating the F58I substitution (Fig. 2g). In the human P2Y2 receptor homology model docked with ATP, Q165<sup>4,57</sup> is oriented toward the adenine ring of ATP. These findings suggest that the Q165H substitution favors receptor interactions with ATP that result in increased downstream signaling. These effects of the Q165H substitution are further amplified by increased expression of the human P2Y2 receptor driven by F58I or synonymous substitutions. In summary, none of the tested combinations of mutations were superior to the original set of ten selected human P2Y2 receptor mutants, which provided a range of improved sensitivity to physiological concentrations of eATP associated with inflammation.

**eATP-driven ATPase activity in engineered yeasts.** We next incorporated a therapeutic response element in the yeast synthetic gene circuit responsive to eATP. We focused on apyrases, which hydrolyze pro-inflammatory eATP and participate in its conversion into immunosuppressive adenosine<sup>14</sup>. We selected the apyrase encoded by *RROP1* in potato (*Solanum tuberosum*), which has potent ATPase activity and reduces inflammation in a mouse model of IBD when delivered intraperitoneally<sup>23</sup>, and apyrase from wheat *Triticum urartu* (referred to as TUAP1 here), based on its sequence homology to *RROP1* in apyrase regions and reported high levels of activity<sup>29</sup> (Extended Data Fig. 3a). To enable secretion by yeast, we replaced

the endogenous apyrase N-terminal signal peptide with the MF $\alpha$ 1 signal peptide and added a C-terminal hemagglutinin (HA) tag to monitor expression (Fig. 3a).

We detected multiple protein bands when apyrase was expressed by yeast, suggesting that it may be partially degraded (Fig. 3b). However, commercially available potato apyrase has bands at 15 and 25 kDa, and apyrases expressed in yeast are glycosylated, resulting in multiple protein bands<sup>30</sup>. Culture supernatants from yeasts expressing *RROP1* (BS029) or TUAP1 (BS030) showed ATPase activity equivalent to ~280 pM or <62.5 pM, respectively, of commercial apyrase per  $\mu$ l raw supernatant (Fig. 3c and Extended Data Fig. 3b,c) (Quantification of secreted ATPase activity in the Methods). Thus, we chose *RROP1* as a therapeutic response element for subsequent studies.

We then co-introduced sensing (P2Y2) and responding (*RROP1*) elements into the genome of the same yeast strains using a CRISPR–Cas9-based approach. We selected six human P2Y2 receptor mutants for integration into the yeast genome based on their low EC<sub>50</sub>, high dynamic range and high maximum activation. We also removed the hygromycin (Hyg)<sup>B</sup> selection marker from the yeast genome to ensure that the final strain would not contain an antibiotic resistance gene while retaining uracil auxotrophy, an important consideration for biocontainment and safety of an engineered microbe.

eATP induced ATPase enzymatic activity in a dose-dependent manner in culture supernatants from yeast strains containing the P2Y2–*RROP1* gene circuit (Fig. 3d,e) (Induction of apyrase secretion with ATP in the Methods). Moreover, ATPase activity was higher in yeast strains expressing engineered P2Y2 receptors than that in the one expressing the WT receptor. For example, at 125  $\mu$ M ATP, we detected a 2.2- to 4.7-fold increase in ATPase activity in yeast strains harboring engineered P2Y2 receptors, while a 1.7- to 2.5-fold increase was detected at the maximum eATP concentration (Supplementary Table 5).

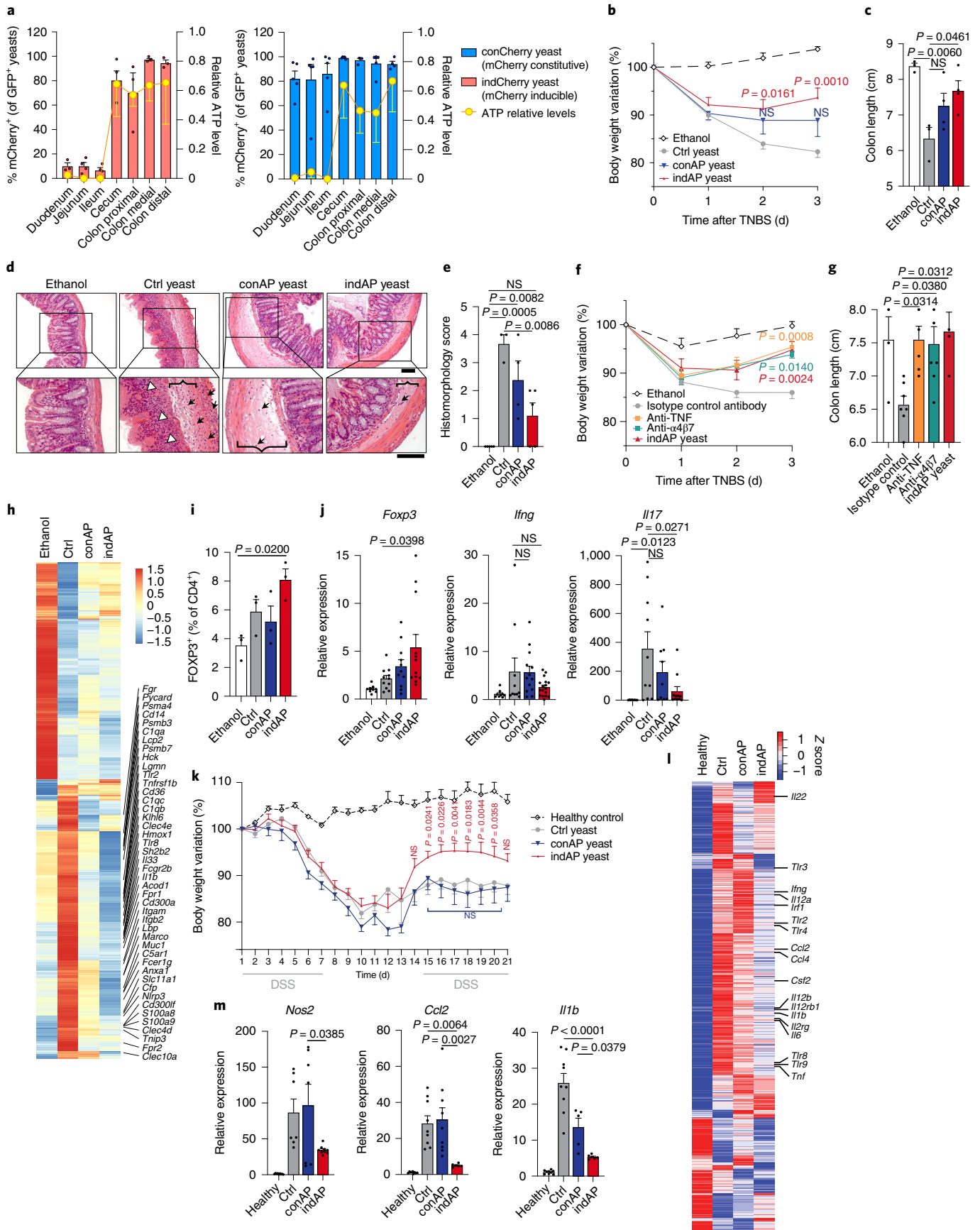
We estimated the theoretical maximum activity of secreted ATPase with a yeast strain constitutively overexpressing *RROP1* (strain BS029, ‘constitutive apyrase’ or conAP). After culturing yeast with 500  $\mu$ M ATP, strains harboring engineered P2Y2 receptor mutant proteins showed 45–69% of the ATPase activity of conAP, while WT P2Y2 showed only 27%. Collectively, these findings show that, through the combination of directed evolution and gene-circuit engineering, we generated yeast strains that secrete functional ATPase in response to physiological levels of eATP.

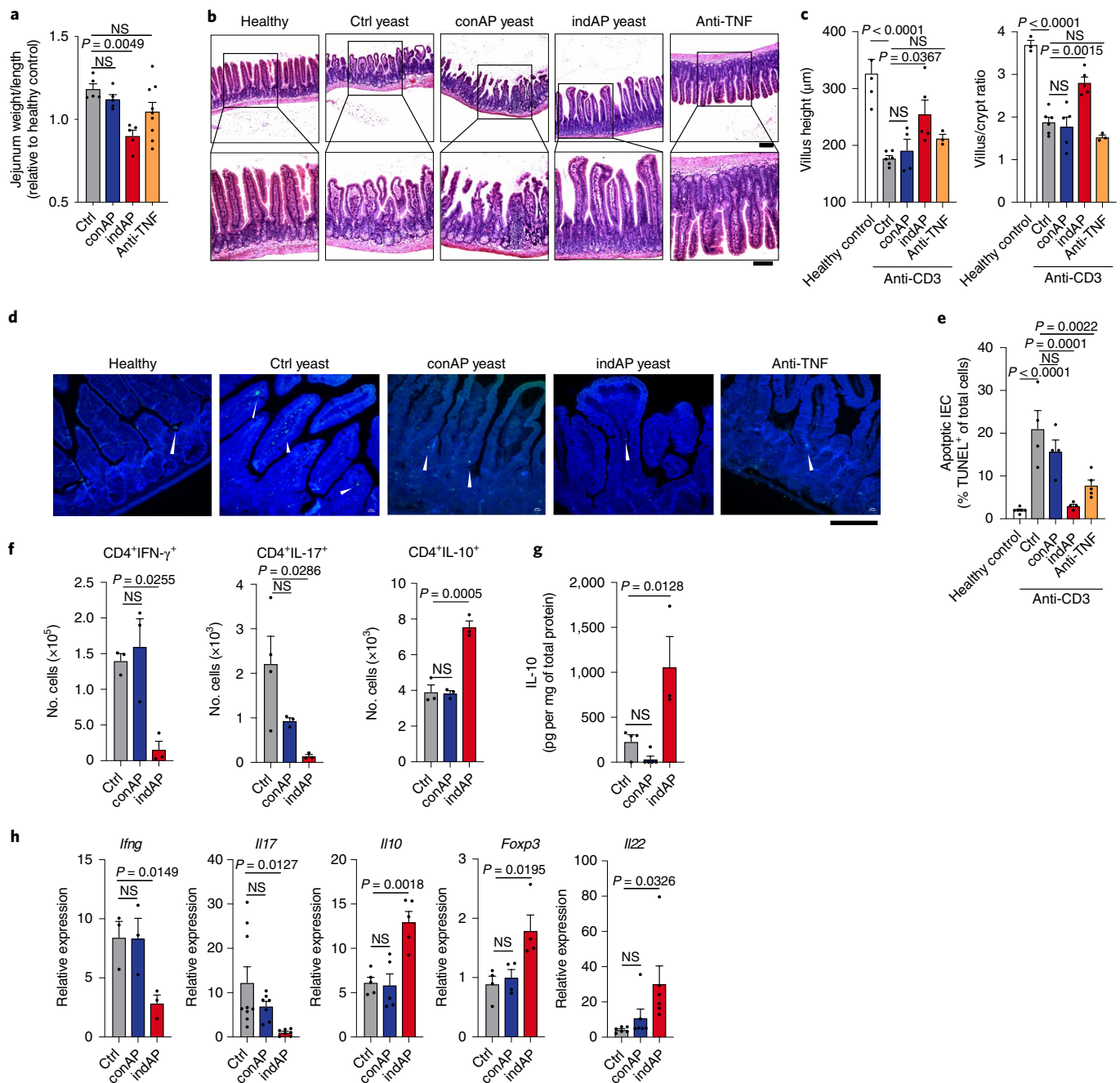
**eATP-responsive yeast ameliorate intestinal inflammation.** We then evaluated the anti-inflammatory activity of the APTM-3 engineered yeast strain that expresses apyrase in an eATP-dependent manner

**Fig. 4 | eATP-responsive engineered yeasts ameliorate chemically induced colitis.** **a**, ATP levels and mCherry<sup>+</sup> yeasts in the fecal content of each portion of the gut from TNBS-treated mice 2 h after administration of eATP-inducible indCherry KG (left) or conCherry KG (constitutive mCherry) (right).  $n = 5$  mice for conCherry KG and  $n = 4$  mice for indCherry KG. **b**, Changes in body weight following TNBS rectal administration.  $n = 10$  mice for ethanol,  $n = 8$  mice for conAP and  $n = 9$  otherwise. **c**, Colon length of mice from experimental groups in **b**.  $n = 3$  mice for ethanol and Ctrl and  $n = 4$  mice for conAP and indAP. **d**, H&E staining at 200 $\times$  (top) and 400 $\times$  (bottom) magnifications. One representative colon section per group is shown. Open arrowheads, immune cell infiltrates in the mucosa with structure disruption. Black arrows, immune cells infiltration at the submucosa. Black brackets, edematous submucosa. Scale bars, 100  $\mu$ m. Data are representative of three independent experiments. **e**, Histomorphology disease scores of mice from groups as in **b** (Histological evaluation in the Methods).  $n = 3$  mice for Ctrl,  $n = 4$  mice for conAP and  $n = 5$  otherwise. **f**, Changes in body weight following TNBS rectal administration.  $n = 4$  mice for ethanol,  $n = 5$  otherwise. **g**, Colon length in mice shown in **f**.  $n = 4$  mice for ethanol and indAP yeasts,  $n = 7$  for isotype control antibody and anti- $\alpha$ 4 $\beta$ 7 antibody and  $n = 6$  for anti-TNF antibody. **h**, RNA-seq analysis of colon samples from mice of groups in **b**. Heatmap of differentially expressed genes. **i**, FOXP3<sup>+</sup> T<sub>reg</sub> cells in mesenteric lymph nodes of groups in **b**.  $n = 3$  samples. **j**, Quantitative (q)PCR for *Foxp3*, *Ifng* and *Il17* mRNA expression in the colon of groups in **b**.  $n = 9$ –17 samples (Source data). **k**, Changes in body weight during DSS-induced colitis in mice treated as in **b**.  $n = 5$  mice for ethanol,  $n = 10$  mice for conAP and  $n = 13$  otherwise. **l**, NanoString gene-expression analysis in the colon from the groups in **k**. Heatmap of differentially expressed genes. Data are representative of two independent experiments with three pooled mice per group. **m**, qPCR for *Nos2*, *Ccl2* and *Il1b* expression in the colon from the groups in **k**.  $n = 5$ –9 samples (Source data). Data are representative of three independent experiments. *P* values are indicated. Two-way ANOVA, Tukey’s post hoc test for **b**, **f**, **k**. One-way ANOVA, Tukey’s or Sidak’s post hoc tests for selected multiple comparisons for **c**, **e**, **g**, **i**, **j**. Bars represent the mean, and error bars represent s.e.m.

(‘inducible apyrase’ strain or indAP). We selected this yeast strain because it secretes low levels of apyrase when unstimulated, yet its ATPase activity was greater than or similar to ATPase activity detected

in other engineered P2Y2–RROP1 strains (Fig. 3e). In addition, its increased sensitivity to eATP can be directly connected to a single substitution in P2Y2 with a known mechanism of action (N116S<sup>35</sup>).





**Fig. 5 | eATP-responsive engineered yeasts ameliorate anti-CD3 antibody-induced enteritis. a**, Jejunal weight/length ratio measured 3 h after anti-CD3 monoclonal antibody administration.  $n = 9$  mice for the anti-TNF antibody and  $n = 5$  otherwise. **b**, H&E staining at 100 $\times$  (top) and 200 $\times$  (bottom) total magnifications. A representative jejunum section of each group 24 h after injection with the anti-CD3 antibody is shown. Scale bars, 100  $\mu\text{m}$ . **c**, Villus height and ratio of villus height/crypt depth were measured from H&E staining images.  $n = 6$  mice for Ctrl,  $n = 3$  mice for anti-TNF antibody and  $n = 4$  otherwise. **d**, Terminal uridine nick-end labeling (TUNEL) analysis of jejunum tissue 24 h after injection. Scale bar, 100  $\mu\text{m}$ ; 400 $\times$  total magnification. **e**, Average number of TUNEL<sup>+</sup> cells per field. Ten fields per mouse were quantified. One-way ANOVA followed by Tukey's post hoc test.  $n = 6$  mice for healthy control,  $n = 5$  mice for anti-TNF antibody and  $n = 4$  otherwise. IEC, intestinal epithelial cells. **f-h**, Intestinal T cells in the jejunum analyzed 24 h after injection with the anti-CD3 antibody. **f**, Jejunal infiltrating IFN- $\gamma$ <sup>+</sup>, IL-17<sup>+</sup> and IL-10<sup>+</sup> CD4<sup>+</sup> T cells analyzed by flow cytometry.  $n = 3$  mice. **g**, IL-10 in the jejunum quantified by ELISA.  $n = 4$  mice for Ctrl,  $n = 5$  mice for conAP and  $n = 3$  mice for indAP. **h**, qPCR for *Ifng*, *Il17*, *Il10*, *Foxp3* and *Il22* expression in the jejunum.  $n = 3$  mice per group for *Ifng*,  $n = 7$  for *Il17*,  $n = 5$  for *Il10*,  $n = 4$  for *Foxp3* and  $n = 6$  for *Il22*. All presented data are representative of two independent experiments.  $P$  values are indicated. Bars represent the mean, and error bars represent s.e.m. One-way ANOVA followed by Tukey's post hoc test for **a, c, e-h**.

We first evaluated the viability of the engineered yeasts in the murine digestive tract. To address this point, we incorporated an antibiotic resistance cassette with a constitutive GFP reporter ('KG' strains) into the CB008 parent strain (from now on named

'Ctrl'), conAP and indAP engineered yeast strains to generate G418-resistant strains, levels of which can be easily quantified in fecal cultures (Yeast culture from mouse feces in the Methods). We detected viable antibiotic-resistant yeasts in feces at 6 h, but not at

24 or 48 h, after oral administration of Ctrl KG, conAP KG or indAP KG yeasts, suggesting that these probiotics are viable in the gut but do not colonize it (Extended Data Fig. 4a).

Increased local eATP levels are associated with intestinal inflammation<sup>11,13,24</sup> (Extended Data Fig. 4b). Thus, to analyze the activation of P2Y2 signaling in engineered yeasts during experimental colitis, we used a strain in which the activation of mutant TM-3 P2Y2 induces mCherry expression (indCherry KG) or a constitutively mCherry-expressing control strain (conCherry KG) (Fig. 1b). Both strains constitutively express GFP, allowing for easy detection in the gut content (Extended Data Fig. 4c). Following oral administration, we detected mCherry expression in indCherry KG engineered yeasts in the cecum and colon, concomitant with increased local eATP levels in trinitrobenzenesulfonic acid (TNBS)-treated mice (Fig. 4a). mCherry expression was not induced in the indCherry KG strain administered to naive mice, in which eATP levels were not locally increased (Extended Data Fig. 4d). Conversely, mCherry expression was detected throughout the gastrointestinal tract of mice that received the conCherry KG strain, regardless of eATP levels (Fig. 4a). Moreover, when we compared mCherry expression under the control of WT or mutant TM-3 P2Y2 in vivo, we detected higher mCherry expression levels in yeasts expressing the mutant TM-3 P2Y2 (indCherry KG versus WTCherry KG strains), highlighting the importance of increased P2Y2 sensitivity for the detection of eATP levels associated with intestinal inflammation (Extended Data Fig. 4e). In sum, these data indicate that the engineered yeast are viable in the digestive tract and that the TM-3 P2Y2 mutant responds in vivo to eATP levels associated with intestinal inflammation.

We then evaluated the therapeutic value of the engineered yeasts administered orally in a model of TNBS-induced colitis (see TNBS-induced mouse colitis model in the Methods for more details). We administered daily the indAP yeast strain, in which apyrase is induced following activation of mutant TM-3 P2Y2 by eATP. As controls, we used the parent strain Ctrl and the conAP strain, which constitutively express apyrase. indAP administration ameliorated TNBS-induced colitis, as indicated by evaluation of weight loss, colon shortening and histological analysis of intestinal pathology (Fig. 4b–e). Of note, the beneficial effects of indAP yeasts in TNBS-induced colitis were comparable to those achieved by the administration of anti-tumor necrosis factor (TNF) or anti- $\alpha 4\beta 7$  integrin blocking antibodies, similar to those used to treat patients with IBD (Fig. 4f,g). Due to their systemic and unspecific nature, these treatments present substantial side effects, which could potentially be overcome with the engineered yeast strains presented in this study<sup>31–33</sup>.

We detected decreased expression of pro-inflammatory genes in colon samples from mice treated with apyrase-producing yeast strains conAP and indAP; these effects were more pronounced in the indAP group (Fig. 4h). Indeed, treatment with the indAP strain,

but not with conAP, led to increased numbers of forkhead box (FOX) P3<sup>+</sup> T<sub>reg</sub> cells in mesenteric lymph nodes and reduced the expression of pro-inflammatory cytokines interferon (IFN)- $\gamma$  and interleukin (IL)-17, associated with intestinal inflammation<sup>34,35</sup> (Fig. 4i,j).

To further evaluate the therapeutic potential of engineered apyrase-expressing yeasts, we used the dextran sulfate sodium (DSS)-induced model of colitis (refer to DSS-induced mouse colitis model in the Methods for details). Treatment with indAP, but not with conAP, interfered with the weight loss associated with DSS-induced colitis (Fig. 4k). Moreover, transcriptional analysis of colon samples revealed that indAP treatment resulted in decreased expression of genes associated with IBD and intestinal inflammation<sup>34–36</sup> (Fig. 4l,m). Pretreatment with indAP for 3 d before the start of DSS administration did not result in significant disease amelioration (Extended Data Fig. 4f), suggesting that indAP predominantly arrests inflammation and T cell-driven pathology taking place at late stages of the disease model<sup>37</sup>.

Finally, we tested indAP in the mouse model of enteritis induced by administration of an anti-CD3 T cell-activating monoclonal antibody<sup>38,39</sup> (Anti-CD3 antibody-induced enteritis in the Methods). This model is characterized by T cell-driven inflammation in the small intestine and fluid accumulation in the jejunal lumen 3 h after anti-CD3 antibody administration, a phenomenon known as enteropooling<sup>38,39</sup>. The administration of indAP, but not that of conAP, significantly reduced enteropooling (Fig. 5a). indAP administration also decreased jejunal mucosal pathology, as indicated by decreased villous disruption and intestinal epithelial cell apoptosis (Fig. 5b–e). Moreover, indAP administration led to a decrease in jejunum-infiltrating T helper T<sub>H</sub>1 and T<sub>H</sub>17 cells, concomitant with increased numbers of IL-10-producing CD4<sup>+</sup> T cells (Fig. 5f). In agreement with these findings, indAP administration was associated with increased IL-10 levels in jejunum samples (Fig. 5g,h). Of note, TNF blockade decreased apoptosis as previously described<sup>40</sup> (Fig. 5d,e) but did not significantly ameliorate other parameters associated with anti-CD3 antibody-induced pathology (Fig. 5a,c). In sum, these findings demonstrate that eATP-responsive yeasts harboring a synthetic P2Y2–RROP1 gene circuit ameliorate intestinal inflammation.

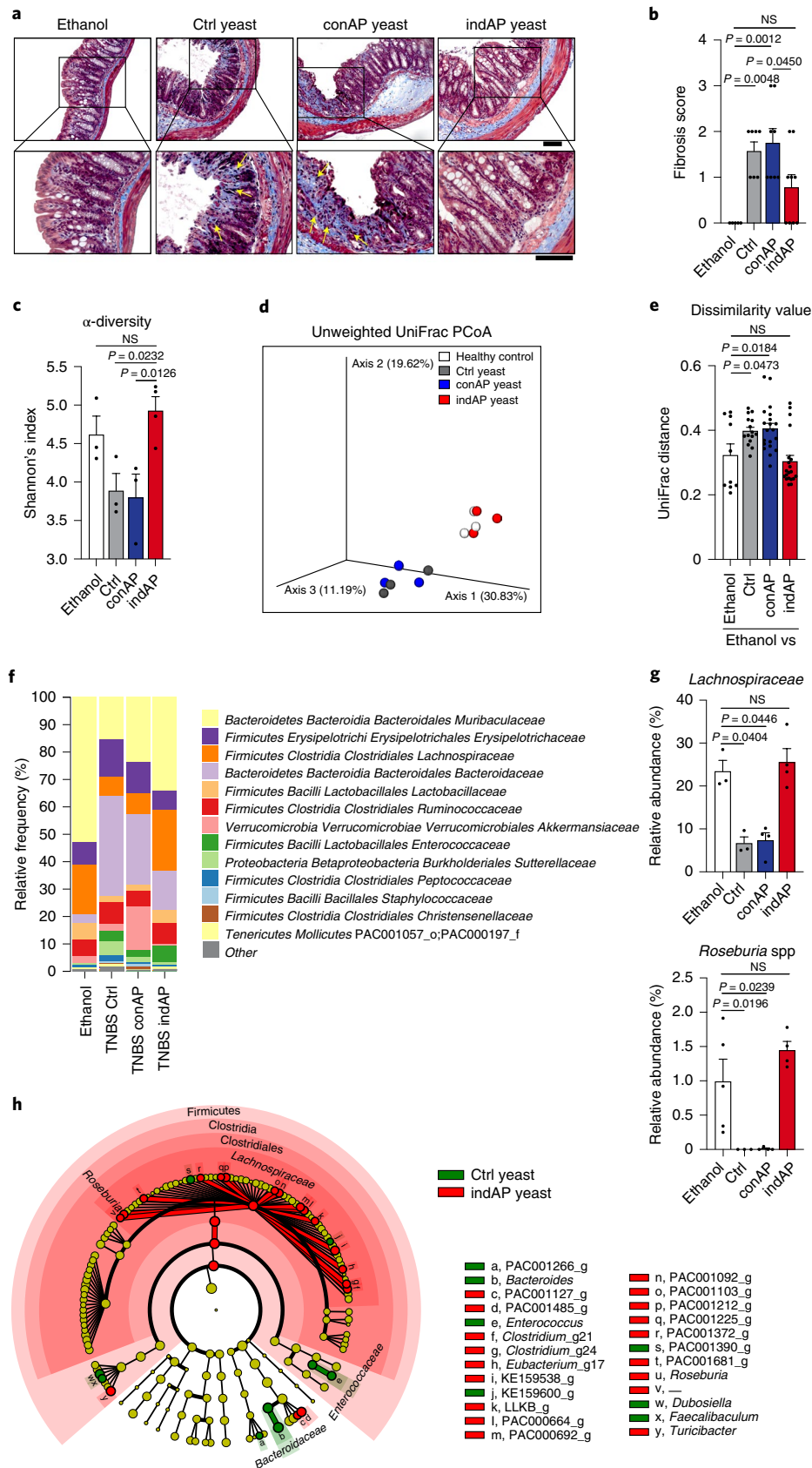
**eATP-responsive yeasts limit colitis-associated fibrosis and dysbiosis.** Although yeast strains constitutively expressing high levels of apyrase have anti-inflammatory effects, they may also promote additional pathogenic responses. Indeed, we detected fibrotic lesions in the colon of mice undergoing TNBS-induced colitis treated with the Ctrl strain and also with constitutive apyrase-expressing conAP yeast strains. However, we detected a significant reduction in fibrosis in mice treated with the eATP-inducible indAP engineered yeast strain (Fig. 6a,b). These findings suggest that controlled modulation of purinergic signaling is needed to manage intestinal inflammation and avoid unwanted deleterious side effects.

**Fig. 6 | eATP-responsive engineered yeast probiotics limit fibrosis and dysbiosis.** **a**, Masson's trichrome staining for fibrosis on sections from mice with TNBS-induced colitis or ethanol-treated controls at 200 $\times$  (top) and 400 $\times$  (bottom) magnifications. A representative colon section of each group is shown. Fibrotic regions are stained in blue and highlighted with yellow arrows. Scale bars, 100  $\mu$ m. Data are representative of two independent experiments. **b**, Histology fibrosis scores of mice from groups from **a**.  $n = 5$  samples for ethanol,  $n = 7$  samples for Ctrl,  $n = 8$  samples for conAP and  $n = 9$  samples for indAP. One-way ANOVA followed by Tukey's post hoc test. **c–h**, High-throughput gene-sequencing analysis of the V4 region of the microbial 16S rRNA gene in fecal samples from mice with TNBS-induced colitis or ethanol-treated controls. **c**,  $\alpha$ -diversity of the fecal microbiome. Shannon's index, which measures evenness and richness of the ecosystem, was calculated at a sequencing depth of 4,000 reads per sample.  $n = 4$  samples for indAP and  $n = 3$  otherwise. Kruskal–Wallis nonparametric ANOVA test. **d, e**,  $\beta$ -diversity. **d**, Principal-coordinate analysis (PCoA) based on unweighted UniFrac metrics. **e**, Pairwise unweighted UniFrac distances to the ethanol control group. PERMANOVA analysis ( $n = 10$  permutations for ethanol,  $n = 15$  permutations for Ctrl,  $n = 20$  permutations for conAP and indAP). **f**, Relative abundance of bacteria classified at a family-level taxonomy. **g**, Relative abundance of the *Lachnospiraceae* family and one of its genera, *Roseburia*.  $n = 3$  samples for ethanol and Ctrl and  $n = 4$  samples for conAP and indAP. Kruskal–Wallis nonparametric ANOVA test. **h**, Linear discriminant analysis effect size,  $P < 0.05$  for the indAP versus Ctrl comparison. Each cladogram represents all taxa detected at  $>0.1\%$ , shown at the kingdom through the genus phylogenetic level. A yellow circle depicts taxa present but not enriched. Red circles are enriched in indAP, and green circles are enriched in Ctrl.  $P$  values are indicated. Bars represent the mean, and error bars represent s.e.m.



Probiotics engineered to act in an inducible and localized manner are likely to minimize disturbances of the gut microbiome. To investigate whether constitutive versus inducible apyrase production

by engineered yeast strains differ in their effects on the gut microbiome, we performed 16S rRNA sequencing of fecal samples from healthy mice or mice with TNBS-induced colitis treated with



engineered yeasts (refer to Microbiome (16S) sequencing and analysis in the Methods for extended details). In agreement with previous reports<sup>41</sup>, induction of colitis with TNBS reduced microbiome diversity within each sample, as indicated by analysis of the Shannon entropy index of  $\alpha$ -diversity (Fig. 6c). A similar reduction in microbiome diversity was detected after treatment with the conAP yeast strain. However, treatment with the indAP engineered yeast strain expressing inducible apyrase resulted in microbiome diversity levels similar to those detected in naive mice (Fig. 6c).

We then analyzed  $\beta$ -diversity, which measures differences in microbiome composition between samples (Fig. 6d,e), revealing significant differences between the control group and TNBS-treated mice treated with either Ctrl or constitutive apyrase conAP yeast strains. Strikingly,  $\beta$ -diversity analysis revealed that TNBS-treated mice treated with the indAP engineered strain harbored a microbiome similar to that of mice in which colitis had not been induced, suggesting that the engineered yeast strain in which apyrase expression is induced by eATP re-establishes a healthy microbiome (Fig. 6d,e).

Finally, we analyzed the taxonomic composition of the microbiome in the different treatment groups. Several taxa of commensal bacteria were shown to be decreased in IBD and ameliorate intestinal inflammation<sup>3,5</sup>. For example, *Clostridium* cluster XIVa, associated with induction of T<sub>reg</sub> cells, is consistently depleted in people with IBD and acute colitis<sup>42</sup>. We found that the *Lachnospiraceae* family, included in *Clostridium* cluster XIVa, was significantly reduced in TNBS-treated mice treated with Ctrl and conAP yeast strains but not in TNBS-treated mice treated with the indAP strain (Fig. 6f–h). Moreover, within the *Lachnospiraceae* family, the *Roseburia* genus was decreased in mice treated with Ctrl and conAP yeast strains but not in indAP-treated TNBS-treated mice. Of note, *Roseburia* spp. were shown to promote T<sub>reg</sub> development through a butyrate-dependent mechanism<sup>43</sup>. In sum, these findings suggest that inducible production of apyrase by the indAP engineered yeast strain enables anti-inflammatory effects of eATP depletion and adenosine production, without unwanted side effects associated with fibrosis and microbiome dysregulation.

## Discussion

The modulation of the eATP–adenosine balance is an attractive strategy to treat inflammation. However, clinical application of this approach requires inducible systems to minimize unwanted side effects such as immunosuppression and fibrosis<sup>14,16,18</sup>. Here we report the development of engineered yeasts that, in response to the metabolite eATP produced in the inflamed gut, secrete apyrase, which depletes pro-inflammatory eATP and promotes the generation of immunosuppressive adenosine. This approach suppresses intestinal inflammation, while reducing fibrosis and promoting the recovery of a healthy microbiome, minimizing the dysbiosis associated with the pathology of IBD and other human disorders<sup>4,5</sup>.

The engineered yeasts that we developed harbor a self-tunable P2Y2–RROP1 gene circuit responsive to pro-inflammatory eATP in a time- and location-specific manner. By engineering the human P2Y2 GPCR, we were able to tune the anti-inflammatory response to physiological eATP levels. Multiple mechanisms contribute to the increase in eATP sensitivity, including increased expression and stabilization of the active receptor conformation. The wide range of solutions selected in response to the challenge imposed by our directed evolution strategy highlights the intrinsic plasticity of GPCR signaling networks. The therapeutic yeast platform described herein could be further optimized by making it responsive to multiple stimuli in the inflamed microenvironment using logic gates<sup>44</sup> and also by incorporating multiple anti-inflammatory mechanisms, such as secreting cytokines and blocking antibodies, which may otherwise cause undesired side effects when administered systemically.

The development of therapeutic probiotics is a major area of IBD research. Previous attempts used engineered probiotics expressing therapeutic proteins in an uncontrolled manner, based on plasmid systems that require constant selection pressure and present the risk of horizontal transfer to other microbes<sup>10,45</sup>. To overcome these limitations, we applied directed evolution and CRISPR–Cas9 to engineer yeast strains with a gene circuit that produces apyrase in response to eATP levels, delivering a probiotic-based dynamic anti-inflammatory response in the inflamed tissue microenvironment. Moreover, we integrated this engineered gene circuit into the yeast genome to avoid the use of antibiotic selection markers, while maintaining uracil auxotrophy for biocontainment, in agreement with Food and Drug Administration guidelines on live biotherapeutic organisms (docket number FDA-2010-D-0500).

*S. cerevisiae* has not been commonly used as a probiotic. However, *S. cerevisiae* strains are a component of the healthy microbiome, which is reduced during IBD<sup>46,47</sup>, and have been associated with physiological training of the immune system<sup>48–51</sup>. Indeed, supplementation with *S. cerevisiae* has been proposed as an anti-inflammatory approach based on its ability to increase IL-10 levels<sup>47</sup>. Notably, natural anti-inflammatory effects of *S. cerevisiae* appear to be strain dependent, as some wild *S. cerevisiae* isolates exacerbate colitis<sup>52,53</sup>, while others ameliorate it<sup>54</sup>. Alternatively, *Saccharomyces boulardii* has been commonly used as a yeast probiotic<sup>19</sup>, but the tools for its genetic manipulation have become available only very recently<sup>55,56</sup>. Thus, the inducible system described in this work may be implemented in *S. cerevisiae*, or it can be transferred to *S. boulardii* to increase its translational potential. However, it is important to consider that the higher survival and growth rate of *S. boulardii* in the gut, when compared to those of *S. cerevisiae*<sup>55,56</sup>, could favor its engraftment, which is not desirable because of the potential for competition with the native gut flora and dysbiosis.

In summary, the engineered yeasts described in this work could represent a step forward in the application of directed evolution and synthetic biology to develop self-tunable anti-inflammatory probiotics. eATP promotes intestinal inflammation in other diseases aside from IBD, such as graft-versus-host disease and irradiation-induced abdominal fibrosis<sup>57,58</sup>. Moreover, the intestinal microbiome controls inflammation at distant body sites such as the central nervous system<sup>59,60</sup>. Thus, control of the intestinal pro-inflammatory–anti-inflammatory balance with engineered yeast probiotics such as the ones described in this work may provide a generalizable therapeutic approach for intestinal inflammation and also for inflammatory disorders targeting other tissues beyond the intestinal system.

## Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41591-021-01390-x>.

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## Methods

**Reporter yeast strains.** All genome modifications of initial yeast strains were conducted using homologous recombination of selectable markers and transformed using a standard lithium acetate transformation method with at least 1 µg linear insert DNA. The parent strain was either CB008 (Ctrl), for constitutive overexpression of fluorescent reporter genes<sup>61</sup>, or BS004, for the P2Y<sub>2</sub>-mCherry or P2Y<sub>2</sub>-apyrase gene circuit<sup>62</sup> (see Supplementary Table 1 for detailed strain genotypes, which were all derived from a laboratory-domesticated haploid strain). *pFUS1*-mCherry was integrated at the *MFA2* locus using plasmid pJW609 containing the KanR marker. *pFUS1* was defined as the 1,636 bp immediately upstream of the *Fus1* start codon, and ~1-kb homology regions were used. *STE2* and *SST2* were targeted for deletion using TRP1 and HygB selectable markers respectively, each with 180 bp of flanking homology regions identical to the sequences flanking the ORE. The five C-terminal amino acids of Gpa1 (KIGII) were replaced with a Gpa1-Gα chimera containing the C-terminal amino acids from the indicated human Gα protein, using plasmid pBS600 containing the selectable marker LEU2 and 800-bp homology regions. The *Candida albicans* *Adh* terminator was used for gene knock-ins to express *pFUS1*-mCherry and Gpa1-Gα. To create a strain that constitutively expressed mCherry, the integration plasmid pJW609 was modified to replace the KanMX marker with *HIS3* from *Candida glabrata*, and sequence coding for p*TDH3*-mCherry was inserted at PspOMI and BamHI sites. The linearized *HIS3*-p*TDH3*-mCherry cassette was transformed into strain Ctrl, and integrations were selected by plating on SC-HIS. To create strains that contained the KanMX selectable marker and constitutively expressed GFP, an integration plasmid was constructed using the MoClo Yeast Toolkit. The resulting plasmid pBS211 contained *HO* locus homology regions, the KanMX marker and a yeast codon-optimized gene coding for sfGFP downstream of p*TDH3* (ref. 21). The linearized KanMX-p*TDH3* sfGFP cassette was transformed into strains described in Supplementary Table 1, and integrations were selected by plating on yeast extract peptone dextrose (YPD)—G418 sulfate (200 µg ml<sup>-1</sup>). All strains were confirmed by PCR and flow cytometry.

**Yeast microscopy.** Yeast strain BS016 expressing the endogenous yeast GPCR Ste2 or a yeast codon-optimized sequence coding for human P2Y<sub>2</sub> (obtained from ATUM) C-terminally tagged with GFP were grown to log phase in SC-URA medium. The centromere plasmid pRS316 was used, containing the endogenous *pSTE2* promoter for Ste2 expression or p*TDH3* for P2Y<sub>2</sub> expression. Restriction enzyme sites were introduced into the sequence coding for an amino acid linker (GGERGS) between the final GPCR residue and first GFP residue. Cells were plated on glass-bottomed dishes (Greiner Bio-One) that had been treated with concanavalin A (Sigma-Aldrich) and then covered with 1 ml SC-URA medium. Cells were imaged using a Leica TCS SP8 confocal microscope and processed using Leica LAS X version 3.1.5.16308 software.

**Flow cytometry evaluation of response to ATP and UTP.** Yeast strain BS016 transformed with a human P2Y<sub>2</sub> gene in the pRS316 p*TDH3* vector was grown in SC-URA liquid medium overnight. The same strain transformed with a plasmid not containing a P2Y<sub>2</sub> sequence (vector) was used as a negative control. Cells were diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.05 in 600 µl SC-URA containing ATP (0–25.6 mM; pH 7.0, Bio Basic) or UTP (0–3.2 mM; pH 7.0, Sigma-Aldrich) and incubated for 6 h at 30 °C with shaking (225 r.p.m.). Cells were then treated with cycloheximide at a final concentration of 10 µg ml<sup>-1</sup>. The mCherry signal from at least 10,000 cells was measured for each sample with a Miltenyi Biotec MACSQuant VYB flow cytometer. The mean mCherry fluorescence was determined using FlowJo. For dose–response assays, data were fitted with the ‘log(agonist) vs. response – Variable slope (four parameters)’ model in Prism (GraphPad). After subtracting the mCherry fluorescence signal of the vector control, fluorescence values were normalized to those from the WT P2Y<sub>2</sub> control used in the same experiment to allow comparisons between experiments performed on different days. The flow cytometry gating strategy is shown in Supplementary Fig. 2a. Data were collected using MACSQuantify version 2.11, and analysis was performed using FlowJo software.

**Directed evolution of the human P2Y<sub>2</sub> receptor.** Error-prone PCR mutagenesis was performed using the Agilent GeneMorph II Random Mutagenesis kit with yeast codon-optimized human P2Y<sub>2</sub> as a template. The desired mutation rate of approximately three mutations per P2Y<sub>2</sub> gene, determined by sequencing 12 randomly selected plasmids (Supplementary Table 2), was achieved by using template DNA (150 ng) and 30 cycles. The randomly mutated sequences were inserted into pRS316 p*TDH3* using AarI-based cloning and transformed into NEB 5-alpha competent *Escherichia coli* cells (New England Biolabs), generating >15,000 individual colonies. Cells were scraped off agar plates and mixed together, and plasmid DNA was extracted (QIAquick Spin Miniprep kit, Qiagen) to create the final plasmid library. The library was transformed into the yeast strain BS016 using a high-efficiency lithium acetate-based method, yielding at least tenfold the number of colonies as the total library size, so that each mutant would be screened multiple times. Transformed cells were incubated overnight and then diluted to an OD<sub>600</sub> of 0.05 in 100 ml fresh SC-URA liquid medium containing 100 µM ATP (pH 7.0, Bio Basic) and incubated for either 18 or 6 h at 30 °C with shaking (225 r.p.m.).

(Extended Data Fig. 2). After brief sonication, 10<sup>6</sup>–10<sup>7</sup> cells were gated by side and forward scatter and sorted for the highest ~1% of mCherry signal using a BD Influx cell sorter (gating strategy is shown in Supplementary Fig. 2b). A total of 174 yeast colonies recovered from various sorting experiments were individually screened for their response to 100 µM UTP or 100 µM ATP or screened with no ligand after a 6-h incubation. Of these colonies, 128 colonies exhibited a response to eATP stronger than the one detected using the WT human P2Y<sub>2</sub> receptor; 163 colonies showed a stronger response to eUTP. Plasmids were isolated from selected colonies by first incubating with zymolyase (BioShop Canada) and then extracting plasmid DNA (QIAquick Spin Miniprep kit, Qiagen). Plasmid DNA was amplified using NEB 5-alpha competent *E. coli* cells (New England Biolabs), plasmid DNA was sequenced, and fresh BS016 yeast cells were transformed for dose–response experiments. Targeted mutations were introduced via PCR following the QuikChange site-directed mutagenesis protocol (Agilent) and using PfuUltra II Fusion HS DNA Polymerase (Agilent).

**Homology modeling of P2Y<sub>2</sub>.** Modeling was conducted as described by Rafehi et al.<sup>63</sup> using the crystal structure of the human P2Y<sub>1</sub> receptor (PDB, 4XNW) bound to the nucleotide antagonist MRS2500 as a template. The sequences of human P2Y<sub>1</sub> and P2Y<sub>2</sub> were aligned using Clustal Omega. As only residues S38–F331 of P2Y<sub>1</sub> were visible in the crystal structure, these were used as the template to generate 500 models of the corresponding P2Y<sub>2</sub> residues L20–L313. Standard MODELLER 9.18 settings were used, maintaining MRS2500 in the models. The generated models were first analyzed based on DOPE and GA341 scores, and the top five models were manually inspected to ensure the natural disulfide bonds were maintained (C25–C278, C106–C183). Next, models were evaluated by ProSA-web<sup>64</sup> and Ramachandran plots<sup>65</sup>, and the final model was selected. ATP was docked to the WT P2Y<sub>2</sub> homology model using the Galaxy7TM web server<sup>66</sup>, which generated ten docked models. The lowest energy model in which the adenine ring of ATP was oriented toward the key Y114 and F261 residues was selected<sup>63</sup>. Publication-quality images were generated with PyMOL (Schrödinger). In addition to residue numbers, in the text we also provide Ballesteros–Weinstein numbering, in which the first number is the transmembrane helix followed by a conserved position across all class A GPCRs<sup>67</sup>.

**Integration of P2Y<sub>2</sub> mutants with CRISPR.** The pCAS plasmid was obtained from Addgene, which expresses Cas9 and a yeast-optimized guide RNA (gRNA)<sup>68</sup>. The gRNA sequence was replaced with an AarI-based multiple cloning site to generate the pCAS AarI plasmid (Extended Data Fig. 5). This enabled the use of AarI, a type IIS restriction enzyme, to insert any gRNA sequence (20 bp) without modifying the required nuclear localization signal or 3' tail of the gRNA. gRNA sequences were designed with CRISPR MultiTargeter<sup>69</sup> and Off-Spotter web servers<sup>70</sup> (Supplementary Table 6). A gene cassette containing an engineered P2Y<sub>2</sub> mutant sequence downstream of the p*TDH3* promoter was assembled in the plasmid pBS600, flanked by 800-bp homology arms for the *SST2* locus. The cassettes were amplified by PCR and transformed into strain BS021 along with plasmid pCAS AarI HygB 1143 as described previously<sup>68</sup>. Colonies were screened for mCherry expression in response to ATP, and P2Y<sub>2</sub> integration was confirmed by sequencing.

**Apyrase genome mining.** Apyrase isolated from the potato species *S. tuberosum* (RROP1; GenBank accession U58597.1) has the highest ATPase activity reported<sup>71</sup>. The BlastPhyMe tool was employed for genome mining of homologous genes, using RROP1 as the initial input sequence. Apyrase from wild einkorn wheat *T. urartu* (named ‘TUAP1’ in our study; GenBank accession KD039156.1) was selected as it had conserved domains known to be required for apyrase function<sup>71</sup> and based on previous reports of wheat apyrase activity<sup>72</sup>. Yeast codon-optimized RROP1 and TUAP1 were modified to contain an N-terminal α-factor signal peptide (first 85 amino acids of yeast Mfα1, lacking the Ste13 cut site) and a C-terminal HA tag (gene synthesis was performed by ATUM).

**Integration of genes encoding apyrases into the genome of P2Y<sub>2</sub> strains.** A gene cassette containing one of the genes encoding apyrase downstream of the *pFUS1* promoter was assembled in the plasmid pBS600. Cassettes were amplified by PCR and transformed into a strain in which P2Y<sub>2</sub> had previously been integrated, along with plasmid pCAS AarI mCherry g664 as described previously<sup>68</sup>. The promoter and terminator of the cassette functioned as homology arms, as sequence coding for mCherry had previously been inserted with *pFUS1* and the *C. albicans* *Adh* terminator at the *MFA2* locus. Colonies were screened for mCherry expression in response to ATP, and integration of the gene encoding apyrase was confirmed by sequencing colonies that did not express mCherry. A second group of gene cassettes was assembled using plasmid pBS603 (pBS600 containing a *HIS3* selection marker) with one of the genes encoding apyrase downstream of the p*TDH3* promoter, flanked by 1-kb homology arms for the *MFA2* locus. Cassettes were amplified by PCR and transformed into strain CB008 (Ctrl) before plating on selective medium to create strains BS029, referred to as conAP (p*TDH3*-RROP1) and BS030 (p*TDH3*-TUAP1).

**Western blot.** Overnight cultures were diluted to an OD<sub>600</sub> of 0.05 in 50 ml YPD. All cultures were incubated for 24 h at 30 °C with shaking (225 r.p.m.). Samples

of lysed cells were resolved on a 10% SDS–PAGE gel (Bio-Rad) and transferred to a PVDF membrane using a Bio-Rad Trans-Blot Turbo transfer system. Membranes were blocked overnight with Odyssey Blocking Buffer (TBS) (LI-COR Biosciences). The following primary antibodies were used: rabbit anti-HA tag (C29F4, Cell Signaling Technology, 1:1,000), mouse anti-PGK (459250, Invitrogen, 1:5,000). After washing, the following secondary antibodies were used: IRDye 680LT Goat anti-Mouse IgG (926-68020, LI-COR Biosciences, 1:5,000), IRDye 800CW Goat anti-Rabbit IgG (926-32211, LI-COR Biosciences, 1:5,000). Bands were visualized with a LI-COR Odyssey CLx infrared imaging system (LI-COR Biosciences) using LI-COR Image Studio version 5.0 software.

**Induction of apyrase secretion with ATP.** Yeast strains containing a *P2Y2* mutant gene and *pFUS1* regulating the expression of *RROP1* apyrase were incubated overnight in YPD medium. Cells were diluted to an  $OD_{600}$  of 0.05 in 2 ml fresh YPD, with 0–500  $\mu$ M ATP (pH 7.0) added. After incubation for 16 h (overnight) at 30 °C with shaking (225 r.p.m.) until an  $OD_{600}$  of 3.5 was reached, 500- $\mu$ l samples were pipetted into 1.5-ml tubes and centrifuged at 2,000g for 5 min to pellet cells. Culture supernatants were then evaluated for ATPase activity.

**Quantification of secreted ATPase activity.** The amount of ATP remaining following incubation with apyrase was determined by Kinase-Glo Plus luminescence (V3771, Promega). In a white 96-well microplate (655075, Greiner Bio-One) 5  $\mu$ l raw supernatant from yeast cultures at an  $OD_{600}$  of 3.5, to which ATP had been added at the start of culturing to induce expression of apyrase, was mixed with 50  $\mu$ M ATP (pH 7.0) in assay buffer (60 mM HEPES, pH 6.0, 2 mM  $MgCl_2$ , 2 mM  $CaCl_2$ , 1 mM dithiothreitol, 0.1 mg ml<sup>-1</sup> BSA, 0.1 mM EDTA and 0.01% Tween-20) to a final volume of 50  $\mu$ l. The reaction was incubated for 30 min at 30 °C and quenched by adding 50  $\mu$ l Kinase-Glo Plus (V3771, Promega). Luminescence was measured with a Fluoroskan Ascent FL microplate reader (Thermo Fisher Scientific) using Ascent software version 2.6. ATPase activity was compared to that of commercial potato apyrase (A6410, Sigma-Aldrich) incubated with ATP under the same conditions. ‘Percent ATP degraded’ was calculated by comparing ATP levels to 50  $\mu$ M ATP incubated in YPD medium and assay buffer under the same conditions.

**Yeast cultures for in vivo testing.** Yeast strains were cultured in 550 ml or 1 l YPD medium (BioShop Canada) at 30 °C with shaking (225 r.p.m.). G418 sulfate antibiotic (200  $\mu$ g ml<sup>-1</sup>, BioShop Canada) was added to the medium when culturing strains containing the KanMX resistance marker. After 24 h, cultures were centrifuged and yeast were resuspended in fresh YPD to a density of approximately  $2 \times 10^9$  colony-forming units (CFU) ml<sup>-1</sup> (~25-fold concentrated culture), and colony density was confirmed by plating. Yeast were stored as 800- $\mu$ l aliquots at –80 °C for up to 1 year.

**Mice.** Mice were kept in an SPF facility at the Hale Building of Transformative Medicine at the Brigham and Women’s Hospital in accordance with Institutional Animal Care and Use Committee guidelines. Two to five mice were housed per cage under a standard light cycle (12 h:12 h light:dark) at 20–23 °C and ~50% humidity with ad libitum access to water and food. C57BL/6J (Jackson Laboratory, 000664) adult female (for the DSS model and the anti-CD3 antibody enteritis model) or male (for the TNBS model) mice between 8 and 10 weeks of age were used throughout the study. Upon receipt, mice were kept for 1 month in our facility for acclimatization and homogenization of microbiome and then were randomly allocated to the different treatment groups without co-housing of mice treated with different probiotic strains to avoid transfer between mice. All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at Brigham and Women’s Hospital and Harvard Medical School.

**Trinitrobenzenesulfonic acid-induced mouse colitis model.** To induce colitis with TNBS in C57BL/6J mice, males were presensitized 1 week before colitis induction by applying 150  $\mu$ l presensitization TNBS solution (64% acetone (179124, Sigma-Aldrich), 16% olive oil (Sigma-Aldrich, O1514), 20% 50 mg ml<sup>-1</sup> TNBS (picrylsulfonic acid solution, 5%, Sigma-Aldrich, P2297)) to their preshaved backs. One week after, presensitized mice were fasted for 4 h, and, subsequently, 100  $\mu$ l TNBS induction solution (50% ethanol, 50% 50 mg ml<sup>-1</sup> TNBS) was administered rectally. The control group was treated only with 50% ethanol. Mouse weight was monitored daily until the day of the euthanasia 72 h after colitis induction, at the peak of the disease.

**Dextran sodium sulfate-induced mouse colitis model.** IBD was induced by adding 4% DSS salt (DSS colitis grade, MP Biomedicals) to the drinking water. Treatment was maintained for 7 d, and two cycles were performed with a week without treatment in between. After the second cycle of DSS, DSS was removed, and mice were killed. Animal body weight was evaluated daily throughout the study. In some studies, treatment was maintained for 7 d, and mice were killed at day 10 when we were only aiming to analyze the first cycle.

**Anti-CD3 antibody-induced enteritis.** C57BL/6J mice were injected intraperitoneally with 200  $\mu$ g anti-CD3 monoclonal antibody (InVivoPlus

anti-mouse CD3 $\epsilon$ ; BP0001-1, Bio X Cell) and killed at the stated time points. For enteropooling, mice were fasted overnight but allowed to drink water ad libitum before injection with anti-CD3 antibody and sacrificed 3 h after injection. The jejunum was then excised and carefully isolated after ligation at both ends, adherent mesentery was cut off, and jejunum segments were then weighed, their lengths were measured, and weight/length ratios were determined.

**Mouse treatment with yeasts.** Mice treated with either DSS or TNBS were given  $2 \times 10^8$  CFU (100  $\mu$ l of  $2 \times 10^9$  CFU ml<sup>-1</sup>) of the corresponding yeast strain by oral gavage for the whole length of the experiment, meaning from day 0 for mice treated with DSS and from the day of presensitization for mice treated with TNBS. Alternatively, to test a preventive schedule in the DSS model, mice were given the same amount starting 3 d before inducing the disease. The same schedule was followed for anti-CD3 antibody-induced enteritis. For studying yeast culture from feces, mice were gavaged once. For mCherry- and ATP-measurement studies, mice were gavaged for 3 d before the study with  $2 \times 10^8$  CFU of the corresponding yeasts.

**Mouse treatment with monoclonal antibodies.** When stated, mice were injected intraperitoneally with 100  $\mu$ g monoclonal antibodies anti-mouse TNF (BE0244, Bio X Cell) or anti-mouse  $\alpha$ 4 $\beta$ 7 (BE0034, Bio X Cell) or the pertinent isotype controls (BE0089, BE0091, Bio X Cell).

**Yeast culture from mouse feces.** Ctrl, conAP and indAP yeasts expressing the gene conferring resistance to the antibiotic G418 were administered by oral gavage as described above. Feces were collected 6, 24 or 48 h after the gavage, weighed, homogenized in PBS and cultured at 30 °C in YPD agar (Y1500, Sigma-Aldrich) containing 500  $\mu$ g ml<sup>-1</sup> G418 (A1720, Sigma-Aldrich). CFU were quantified after 72 h.

**ATP measurement in fecal content.** To evaluate the amount of ATP in the fecal content, feces from the duodenum, jejunum, ileum, cecum and colon of TNBS-treated mice treated with the corresponding yeast strain were collected 72 h after TNBS induction, 2 h after the last gavage with the yeasts. The fecal content of the corresponding part of the gut was homogenized in PBS, and ATP measurements were performed using the ATP Determination kit (A22066, Molecular Probes) following the manufacturer’s instructions. Data were normalized to the weight of the fecal content and to values from the control sample.

**Detection of mCherry reporter yeast strains in vivo.** To confirm the response of our engineered *P2Y2* mutant to ATP in vivo, reporter yeasts expressing mCherry under control of the TM-3 *P2Y2* mutant or WT *P2Y2* and constitutively expressing GFP were administered as described above to mice with TNBS-induced colitis at the peak of the disease when we expect more ATP to be present in the gut. Content from the specified section of the gut was collected 2 h after the gavage, homogenized in YPD medium (Y1375, Sigma-Aldrich) and cultured overnight. GFP and mCherry expression was measured by flow cytometry in a Fortessa flow cytometer (BD Biosciences), and data analysis was performed using FlowJo 10.7 software.

**Microbiome (16S) sequencing and analysis.** Fecal samples were collected from control mice and mice with TNBS-induced colitis from each respective yeast treatment at the end of the study. DNA was extracted using the DNeasy PowerLyzer PowerSoil kit (12855, Qiagen) following the manufacturer’s instructions. The 16S rRNA gene V4 region was amplified and barcoded by PCR using HotMaster Taq DNA Polymerase and HotMasterMix (10847-708, VWR) and a primer library that contained adaptors for MiSeq sequencing and dual-index barcodes so that PCR products could be pooled. DNA was then quantified using the Quant-iT PicoGreen dsDNA Assay kit (P11496, Thermo Scientific), and 100 ng of each sample was pooled and cleaned up using the QIAquick PCR Purification kit (28104, Qiagen). DNA was requantified after cleanup with the Qubit Fluorometric Quantification kit (Thermo Scientific) and submitted for paired-end 151-bp read sequencing on the Illumina MiSeq instrument at the Harvard Medical School Biopolymers Facility as previously described<sup>72</sup>. Quantitative insights into microbial ecology software 2 (QIIME2) was used for quality filtering and downstream analysis for  $\alpha$ - and  $\beta$ -diversity and for compositional analysis following standardized protocols<sup>73</sup>. Dada2 was used to denoise and filter sequences for quality, to join reads and to dereplicate the reads into amplicon sequence variants. Taxonomic assignment was performed using the RDP classifier built into QIIME2, which was trained on the 16S rRNA V4 region database from the EZBioCloud database<sup>74</sup>. Distances between samples ( $\beta$ -diversity) were calculated using the unweighted UniFrac distance metric that evaluates qualitative differences of microbial taxa, taking into account their phylogenetic relationship<sup>75</sup>. Statistical testing for differential clustering of samples on principal-coordinate analysis plots was performed using the PERMANOVA test using 999 permutations. Significant differences in taxa modulated by control or active yeast treatment were determined by linear discriminant analysis effect size<sup>76</sup>.

**Histological evaluation.** Colonic or jejunal tissue was removed and assessed for histological evaluation blindly upon fixation with Bouin’s solution (HT10132, Sigma-Aldrich). Paraffin-embedded tissues were sectioned, stained with H&E and examined for evidence of colitis or inflammation with a Zeiss Axioskop2 Plus

microscope with AxioCam HRC and AxioVision software. Colitis histology scores (range, 0–6) were calculated based on the presence of lymphomononuclear cell infiltrate (0, absence of inflammatory foci; 1, mild presence of inflammatory foci in mucosa; 2, presence of multiple inflammatory foci in mucosa and submucosa; 3, evidence of transmural infiltration) and intestinal architecture disruption (0, normal architecture; 1, presence of focal erosions; 2, erosions and focal ulcerations; 3, extended ulceration, granulation of tissue and/or pseudopolyps) as previously described<sup>77</sup>. For evaluation of enteritis, the length of villi and depth of crypts were measured, and the villus length/crypt depth ratio was calculated. For analysis of fibrosis, Masson's trichrome staining was performed. The TUNEL assay was used to identify apoptotic cells in the jejunal tissue. Briefly, jejunal samples previously fixed in Bouin's solution and then embedded in paraffin were deparaffinized and rehydrated by subsequent incubations with xylene, 100% ethanol, 96% ethanol, 70% ethanol and PBS. TUNEL staining was performed following the manufacturer's instructions (11684795910, Sigma-Aldrich). Sections were analyzed in a Leica DMi8 fluorescent microscope, and positive cells were quantified using ImageJ software<sup>78</sup>.

**Flow cytometry staining and acquisition.** For jejunal intraepithelial lymphocyte extraction, tissues were excised, cleaned and cut in 1-cm pieces. Pieces were incubated subsequently in PBS with 1 mM dithiothreitol (646563, Sigma-Aldrich), 2.5 mM EDTA (E177, VWR) and 2.5% FBS; PBS with 5 mM EDTA and 5% FBS; and finally digested in RPMI with 5% FBS, 1 mg ml<sup>-1</sup> collagenase VIII (C2139, Sigma-Aldrich) and 10 U ml<sup>-1</sup> DNase (90083, Thermo Fisher Scientific). Samples were then filtered through a 40- $\mu$ m cell strainer to obtain single-cell suspensions. Cell suspensions were prepared from mesenteric lymph nodes by smashing lymph nodes through a 70- $\mu$ m cell strainer. Single-cell suspensions were stimulated with 50 ng ml<sup>-1</sup> phorbol 12-myristate 13-acetate (P1585, Sigma-Aldrich), 1  $\mu$ M ionomycin (I9657, Sigma-Aldrich), GolgiStop (554724, BD Biosciences, 1:1,500) and GolgiPlug (555029, BD Biosciences, 1:1,500) in RPMI 1640 medium (11875119, Life Technologies) containing 10% FBS (10438026, Gibco) and penicillin–streptomycin (15140122, Gibco, 1:100) for 4 h at 37 °C in a 5% CO<sub>2</sub> incubator. Antibodies for flow cytometry were BUV395 anti-mouse CD45 (BD Biosciences, 564279, 30-FL1, 1:100), APC anti-mouse IFN- $\gamma$  (BD Biosciences, 554413, XMGL2, 1:100), PE anti-mouse IL-17a (eBioscience, 12-7177-81, TC11-18H10.1, 1:100), BV421 anti-mouse CD4 (BD Biosciences, 562891, GKL5, 1:100), BV650 anti-mouse CD3 (BD Biosciences, 740530, 17A2, 1:100), FITC anti-mouse FOXP3 (eBioscience, 11-5773-82, FJK-16s, 1:100) and PE/Dazzle594 anti-mouse IL-10 (BioLegend, 505034, JES5-16E3, 1:100). Cells were then analyzed on a Fortessa flow cytometer (BD Biosciences). CD4<sup>+</sup>FOXP3<sup>+</sup> cells were defined as CD3<sup>+</sup>CD4<sup>+</sup>IFN- $\gamma$ IL-17<sup>-</sup>IL-10<sup>-</sup>FOXP3<sup>+</sup>; CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells were defined as CD3<sup>+</sup>CD4<sup>+</sup>IFN- $\gamma$ IL-17<sup>-</sup>IL-10<sup>-</sup>FOXP3<sup>-</sup>; CD4<sup>+</sup>IL-17<sup>+</sup> cells were defined as CD3<sup>+</sup>CD4<sup>+</sup>IFN- $\gamma$ IL-17<sup>+</sup>IL-10<sup>-</sup>FOXP3<sup>-</sup>; CD4<sup>+</sup>IL-10<sup>+</sup> cells were defined as CD3<sup>+</sup>CD4<sup>+</sup>IFN- $\gamma$ IL-17<sup>-</sup>IL-10<sup>+</sup>FOXP3<sup>-</sup>IL-10<sup>+</sup>.

**Enzyme-linked immunosorbent assay.** Jejunal tissue (1 cm) was excised and mechanically disrupted into RIPA buffer (89900, Thermo Scientific) supplemented with Halt protease inhibitor (78441, Thermo Scientific). Supernatants were stored at -80 °C. Levels of the IL-10 cytokine were measured by ELISA (88-7105-88, Invitrogen) following the manufacturer's instructions. Data were normalized to protein content in the supernatants measured by BCA (23235, Thermo Scientific).

**RNA extraction and quantitative PCR.** Twenty mg of the distal colon or from the medial jejunum was flash frozen and later disrupted in TRIzol (Invitrogen). RNA was extracted following the manufacturer's instructions for the miRNeasy kit (Qiagen). When needed to remove DSS from RNA, we further purified mRNA using the Oligotex kit (Qiagen). cDNA was prepared using the High-Capacity RT kit (Applied Biosystems) and used for qPCR. Results were normalized to expression levels of *Gapdh*. All primers and probes were from Applied Biosystems: *Gapdh*, Mm99999915\_g1; *Il17a*, Mm00439618\_m1; *Ifng*, Mm00801778\_m1; *Foxp3*, Mm00475162\_m1; *Ccl2*, Mm00441242\_m1; *Nos2*, Mm00440502\_m1; *Il1b*, Mm00434228\_m1.

**Gene-expression analysis by NanoString.** Total RNA (100 ng) from colon tissue obtained from mice 21 d after the start of DSS administration was analyzed using nCounter Mouse Immunology Panel expression code sets according to the manufacturer's instructions (NanoString Technologies). Data were analyzed using nSolver Analysis software and plotted with Heatmapper<sup>79</sup>. Functional pathway enrichment analysis was conducted using Enrichr. The combined score was calculated as  $c = \ln(P) \times z$ , where  $P$  is the  $P$  value computed using Fisher's exact test and  $z$  is the rank score or  $z$  score computed using a modification to Fisher's exact test in which a  $z$  score for deviation from an expected rank is computed<sup>80</sup>.

**Gene-expression analysis by RNA sequencing.** Total RNA (5 ng) from colon tissue was sent for SMART-seq by the Broad Technology Labs and the Broad Genomics Platform. Processed RNA-seq data were filtered, removing genes with low read counts. Read counts were normalized using TMM normalization, and counts per million were calculated to create a matrix of normalized expression values. The fastq files of each RNA-seq data sample were aligned to the *Mus*

*musculus* GRCh38 transcriptome using Kallisto (version 0.46.1), and the same software was used to quantify alignment results. Differential expression analysis was conducted using DESeq2, and log<sub>2</sub> fold change values were adjusted using apeglm for downstream analysis. The Benjamini–Hochberg method was used for multiple-hypothesis-testing correction. The GSEA analysis was performed using apeglm-adjusted differential expression analysis results. Genes that were differentially expressed with adjusted  $P$  values < 0.05 were analyzed with the Ingenuity Pathway Analysis tool to determine significantly regulated pathways.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability

RNA-seq data were deposited in the GEO database under the accession number GSE152869. Sequencing data from microbiota 16S rRNA were submitted to the NCBI short-read archive under BioProject number PRJNA641709. Source data are provided with this paper.

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### Author contributions

B.M.S., C.G.-V., L.M.S., J.A.d.S.P., A.P., P.H., M.O'B. and S.K.C. performed in vitro and in vivo experiments; Z.L., L.M.C. and C.G.-V. performed bioinformatic analysis;

P.M.M.-V. discussed and interpreted findings; B.M.S., C.G.-V. and F.J.Q. wrote the manuscript with input from co-authors; and B.S.W.C., S.G.P. and F.J.Q. contributed equally to the design and supervision of the study and editing the manuscript.

### Competing interests

B.M.S., C.G.-V., B.S.W.C., S.G.P. and F.J.Q. filed a patent for the use of engineered yeast to treat inflammation. All other authors declare no competing interests.

### Additional information

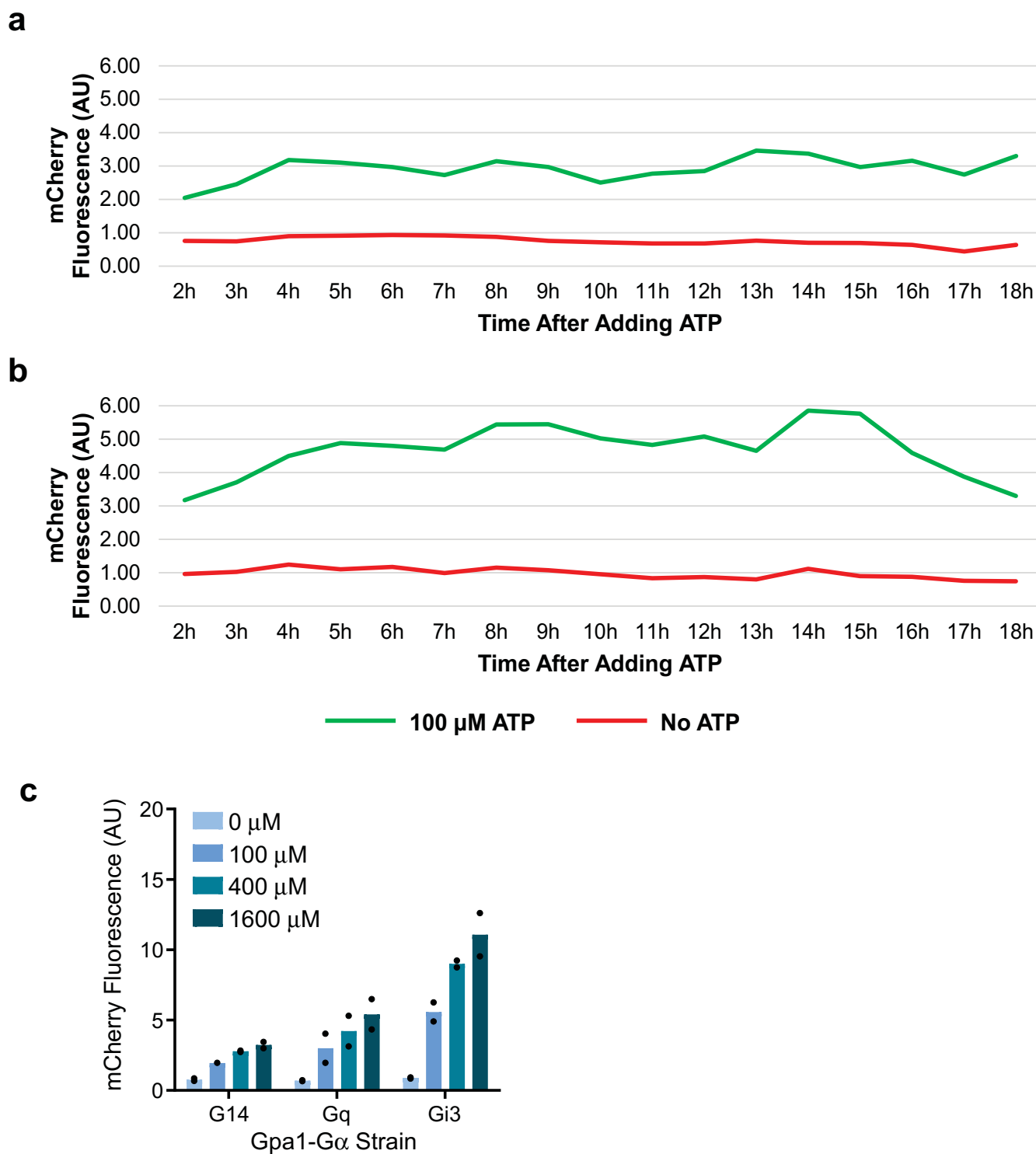
**Extended data** is available for this paper at <https://doi.org/10.1038/s41591-021-01390-x>.

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41591-021-01390-x>.

**Correspondence and requests for materials** should be addressed to F.J.Q.

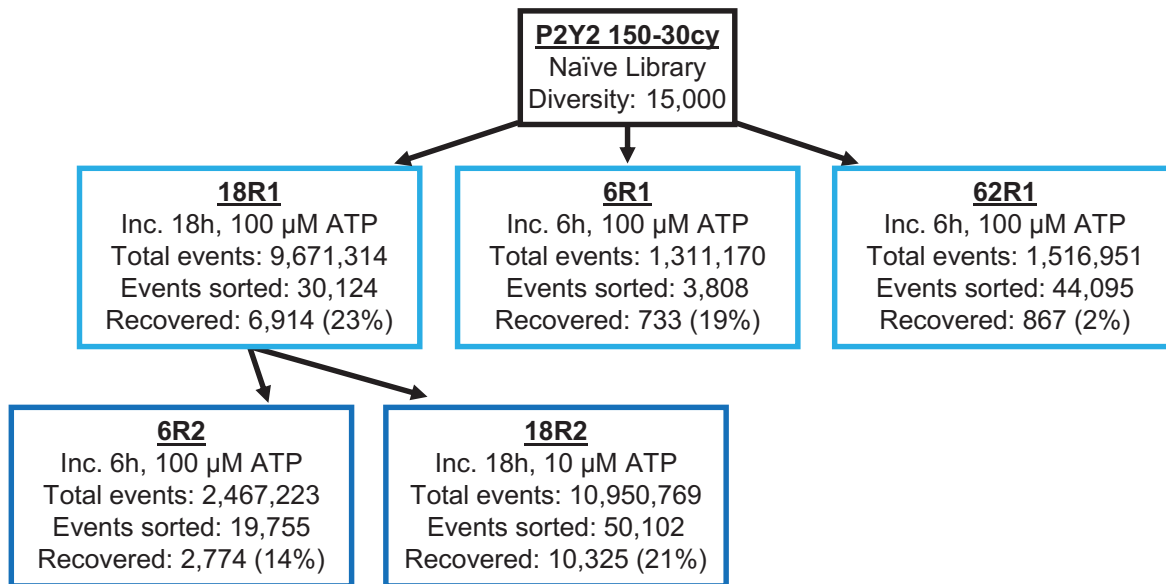
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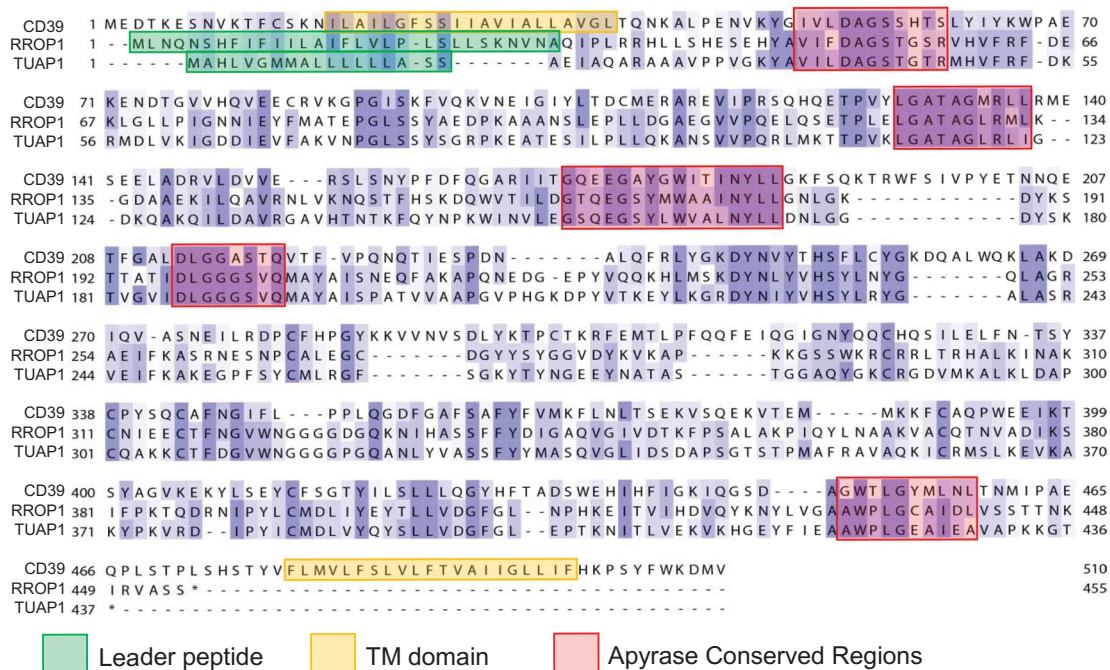
**Extended Data Fig. 1 | Response to eATP over time of engineered mating pathway. a,b,** Yeasts from the BS016 strain transformed with plasmid pRS316 pTDH3 P2Y2 (WT human P2Y2 receptor) were incubated with 100  $\mu$ M ATP in 300  $\mu$ L (**a**) or 5 mL SC-URA media (**b**); and mCherry fluorescence was quantified. Data points represent the mean of 2 colonies. **c,** Engineered mating pathway response to UTP using the wild-type (WT) human P2Y2 receptor and various yeast strains with integrated Gpa1-G $\alpha$  chimeras as follows: BS019 (G14), BS020 (Gq), BS016 (Gi3). Following incubation for 6h with the indicated UTP concentration, the activation of the mating pathway was monitored by quantifying mCherry fluorescence by flow cytometry. Data points represent the mean of 2 colonies.



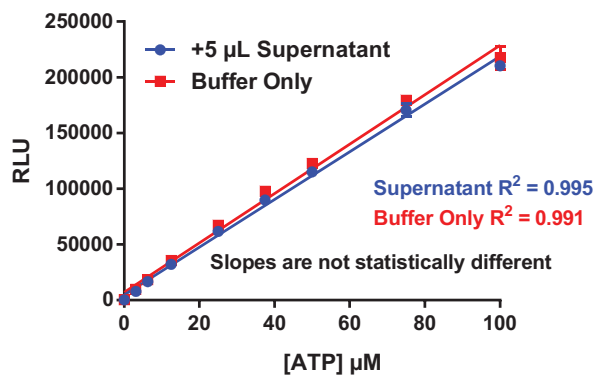


**Extended Data Fig. 2 | Strategy for directed evolution of human P2Y2 receptor.** During each FACS sort the top ~1% of mCherry fluorescence was collected. 'Recovered' refers to the number of yeast colonies obtained after plating sorted cells on selective media.

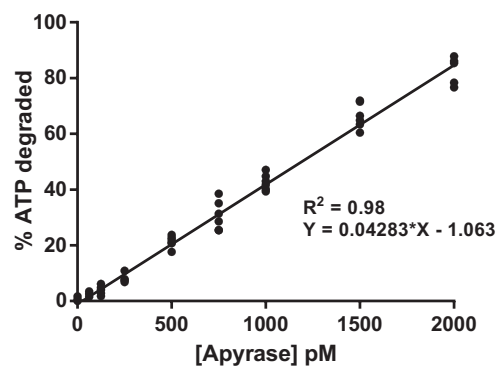
**a**



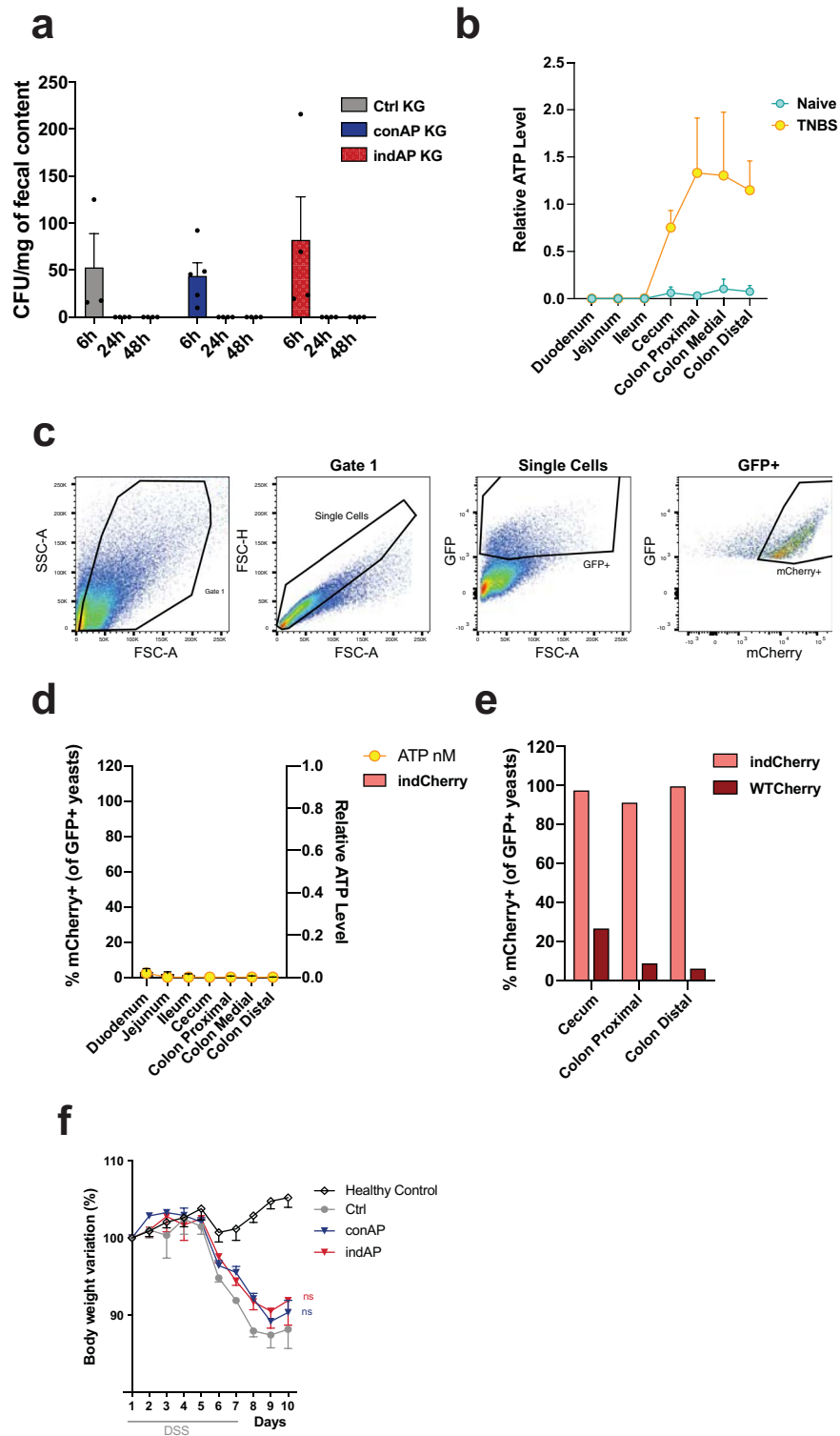
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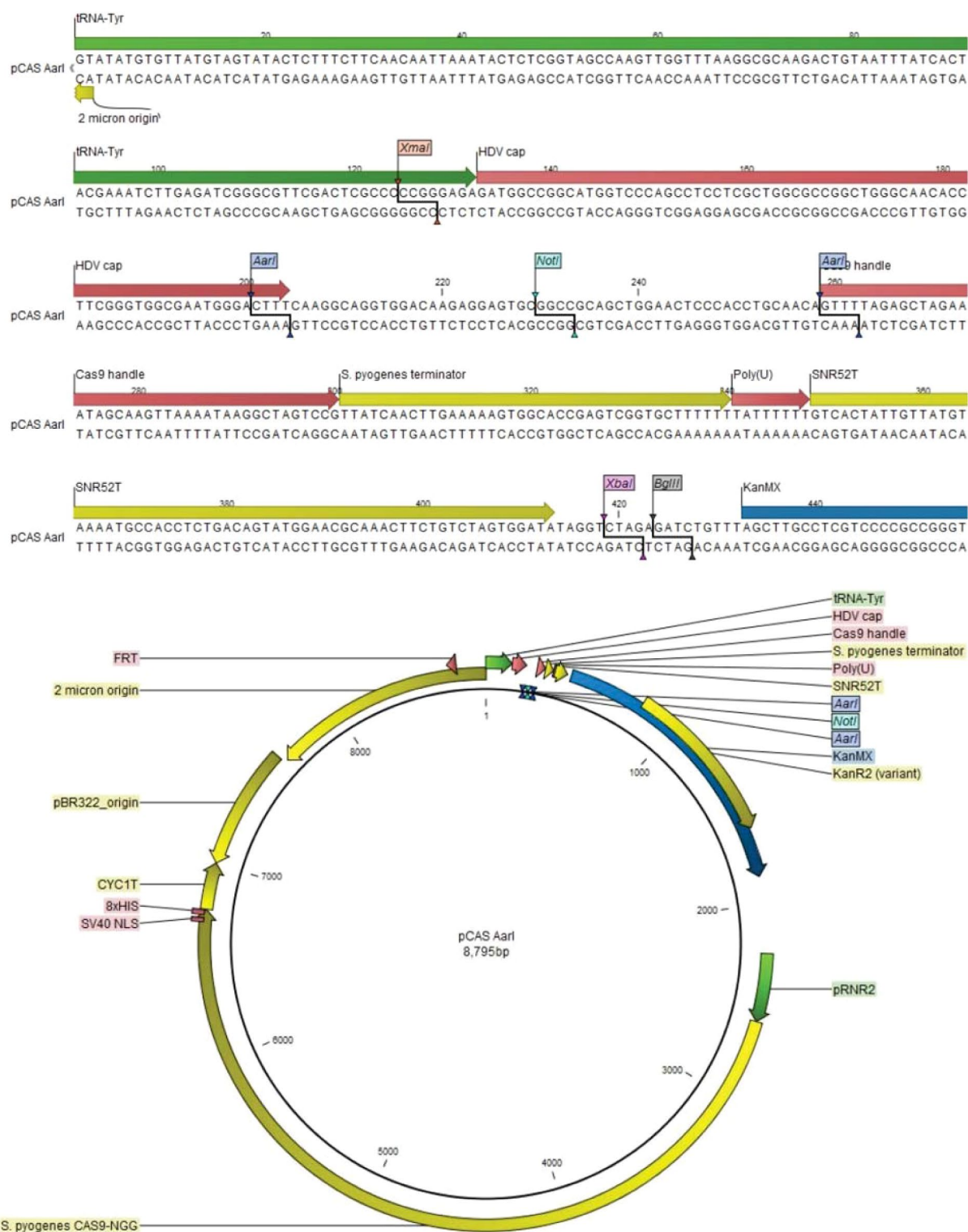
**c**



**Extended Data Fig. 3 | Apyrase Genes and ATP concentration in yeast supernatants.** **a**, Sequence Alignment of Apyrase Genes. Human ENTPD1 (CD39), potato apyrase (RROP1) and wheat apyrase (TUAP1) were aligned using MUSCLE, in the MEGA6 alignment explorer. **b**, Measurement of a known ATP concentration in the presence of 5  $\mu$ L yeast supernatant from strain CB008 (blue) or reaction buffer only (red). Yeast supernatant did not affect the measurement readout.  $n = 3$  samples, error bars represent SD. **c**, To estimate the amount of active apyrase secreted by yeast, 50  $\mu$ M ATP was incubated with the indicated concentration of commercial apyrase for 30 minutes at 30°C, with 5  $\mu$ L supernatant from a culture of strain Ctrl, in a 50  $\mu$ L reaction volume and residual ATP was quantified. No apyrase activity was observed when 31.3 pM commercial apyrase was added.  $n = 6$  samples.



**Extended Data Fig. 4 | Engineered yeasts probiotics are viable in the mouse gut.** **a**, Colony forming units per mg of stool collected 6, 24 and 48h after oral gavage to the mice with either Ctrl KG, conAP KG or indAP KG yeast strains.  $n=3$  Ctrl,  $n=5$  conAP and  $n=4$  indAP samples per group. **b**, ATP relative levels in the specified portions of the gut of naïve and TNBS induced mice.  $n=4$  naïve,  $n=6$  TNBS mice per group. **c**, Gating strategy to measure mCherry positive yeasts in the fecal content. **d**, mCherry positive yeasts (% of total GFP yeast) measured by flow cytometry in the fecal content of the specified portion of the gut after 2h from oral gavage to naïve mice with ATP inducible strain indCherry KG. ATP levels were measured in the same portions of the gut.  $n=3$  samples for cytometry and  $n=8$  for eATP levels. **e**, mCherry positive yeasts (% of total GFP yeast) quantified by flow cytometry in the fecal content of the specified portion of the gut from TNBS treated mice 2 hours after oral gavage with indCherry KG or WTCherry KG yeast strains. **f**, Changes in body weight during the course of DSS-induced colitis in mice treated with engineered yeasts starting 3 days before DSS administration.  $n=4$  mice Healthy control and  $n=3$  otherwise. Two-way ANOVA followed by Tukey's post-hoc test, ns= not significant.



**Extended Data Fig. 5 | Integration of P2Y2 mutants with CRISPR STAR Method. Plasmid pCAS AarI.** Custom multiple cloning site inserted at the XmaI and BglII sites in the pCAS plasmid, obtained from AddGene<sup>68</sup>. Image generated with CLC Sequence Viewer.

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BD FACSDIVA (v.8.0.1); MACSQuantify (v2.11); BD FACS (v1.2.0.142); Li-Cor Image Studio (v5.0); Leica LAS X (v3.1.5.16308); MODELLER (v9.18); Ascent Software (v2.6); AxioVision v4.8.2.0; Promega Glomax Explorer v3.1.0

Data analysis

FlowJo v10.7; nSolver v4.0; QUIME2; Prism v8.4.3; FIJI-ImageJ v2.0; R (v3.4.0); ggplot2 (v3.1.0); FASTQC (v0.11.5); Kallisto (v0.46.1); DESeq2 (1.20.0); STAR v2.7.3; RSEM (v1.3);

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Sample size	No statistical methods were used to predetermine sample sizes. Sample sizes are similar to those used in the field, as previously reported: Sanmarco et al. Nature 2021, Wheeler et al. Nature 2020, Rothhammer et al Nature 2018, Rothhammer et al Nature Medicine 2016, Mayo et al. Nature Medicine 2014
Data exclusions	Data were only formally excluded by formal outlier tests in Prism.
Replication	To ensure replication, all deep sequencing data were repeated in 3-6 mice per group For in vitro and in vivo experiments, experiments were repeated multiple times, at least twice independently as stated in figure legends. All attempts at replication were successful.
Randomization	Samples and mice were randomly allocated into treatment groups.
Blinding	Experimenters were blinded to treatment during colitis disease. Similarly, during data processing of all sequencing data, experimenters were blinded to condition. Otherwise, blinding was not performed, such as during in vitro experiments, where experimenters were required to know the conditions of each well.

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## Antibodies

Antibodies used	BUV395 anti-mouse CD45 (BD Biosciences, #564279, 30-Fl, 1:100); APC anti-mouse IFN $\gamma$ (BD Biosciences, #554413, XMGI.2, 1:100); PE anti-mouse IL-17a (eBioscience, #12-7177-81, TC11-18H10.1, 1:100); BV421 anti-mouse CD4 (BD Biosciences, #562891, GKI.5, 1:100); BV650 anti-mouse CD3 (BD Biosciences #740530, 17A2, 1:100); FITC anti-mouse Foxp3 (eBioscience, #11-5773-82, FJK-16s, 1:100); PE/Dazzle594 anti-mouse IL-10 (Biolegend, #505034, JES5-16E3, 1:100); rabbit anti-HA tag (Cell Signaling Technology, #C29F4, 1:1000); mouse anti-PGK (Invitrogen, #459250, 1:5000); IRDye <sup>®</sup> 680LT Goat anti-Mouse IgG (LI-COR Biosciences, 926-68020, 1:5000); IRDye <sup>®</sup> 800CW Goat anti-Rabbit IgG (LICOR Biosciences, 926-32211, 1:5000); anti-mouse CD3e monoclonal Ab (#BP0001-1 Bio X Cell, 10 mg/kg); Armenian Hamster anti-mouse TNF (#BE0244 Bio X Cell, 5mg/kg); Armenian Hamster IgG # BE0091 Bio X Cell, 5mg/kg); Rat anti-mouse LPAM-1 or integrin $\alpha$ 4beta7 (#BE0034 Bio X Cell, 5mg/kg); Rat IgG2a (#BE0089 Bio X Cell, 5mg/kg). All additional information is readily available on the manufacturer's web site for each antibody
Validation	All antibodies were commercial in origin and validated by the company. Specifically, Flow cytometry antibodies from BD Bioscience, eBioscience/Invitrogen ( <a href="https://www.thermofisher.com/us/en/home/life-science/antibodies/invitrogen-antibody-validation.html">https://www.thermofisher.com/us/en/home/life-science/antibodies/invitrogen-antibody-validation.html</a> ) and Biolegend ( <a href="https://www.biolegend.com/nl-nl/reproducibility">https://www.biolegend.com/nl-nl/reproducibility</a> ) and western blot antibodies from Invitrogen and LI-COR were purified from cell line supernatant using affinity chromatography followed by validation using proper negative and positive controls in functional assays as well as for their mouse reactivity. Western Blot antibody #C29F4 from Cell Signaling Technology ( <a href="https://www.cellsignal.com/about-us/cst-antibody-validation-principles">https://www.cellsignal.com/about-us/cst-antibody-validation-principles</a> ) were purified from serum from animals immunized with a synthetic peptide containing the HA epitope using affinity chromatography followed by validation using proper negative and positive controls in functional assays. Antibodies used in vivo from BioXCell ( <a href="https://bxc.com/the-bio-x-cell-advantage/">https://bxc.com/the-bio-x-cell-advantage/</a> ) were also originated in cell line cultures and each lot validated with a library of recombinant proteins and bioassay to ensure they binds strongly and specifically to their target antigen. Additional information for each specific antibody listed above can be found by entering the antibody product number listed above on the company's web site.

## Animals and other organisms

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Laboratory animals	WT C57BL/6J (The Jackson Laboratory, #000664). Male and female mice were used. Experiments were initiated in 8-12 week old mice.
Wild animals	The study did not involve wild animals.
Field-collected samples	The study did not involve samples collected from the field.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Flow Cytometry

### Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
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### Methodology

#### Sample preparation

For dose-response measurements, yeast cells were incubated overnight, then diluted to OD600 0.05 in 600  $\mu$ L SC-URA media (BioShop Canada, DOM007 with YNB406) containing ATP (0-25.6 mM pH 7.0; Bio Basic, AB0020) or UTP (0-3.2 mM pH 7.0; Sigma-Aldrich, U6625) and incubated for 6 hours at 30°C with shaking (225 rpm). Cells were then treated with cycloheximide (BioShop Canada, CYC003) to a final concentration of 10  $\mu$ g/mL. The mCherry signal of at least 10,000 cells was measured for each sample with a MACSQuant VYB (Miltenyi Biotec).

For cell sorting experiments, yeast cells were incubated overnight, then diluted to OD600 0.05 in 600  $\mu$ L SC-URA media (BioShop Canada, DOM007 with YNB406) containing ATP (0-25.6 mM pH 7.0; Bio Basic, AB0020), and incubated for either 18 or 6 hours at 30°C with shaking (225 rpm). Cells were then treated with cycloheximide (BioShop Canada, CYC003) to a final concentration of 10  $\mu$ g/mL. After brief sonication (two pulses, minimum amplitude), cells were filtered into a sterile tube (35  $\mu$ m filter, Falcon, 352235). One million to ten million cells were then sorted for the highest ~1% of mCherry signal using a BD Influx cell sorter (BD Biosciences).

For Cell suspensions preparation from mesenteric lymph nodes: Animals were euthanized and mesenteric lymph nodes extracted after surgical excision in the abdomen. Tissue was mechanically dissociated and filtered through a 70  $\mu$ m cell strainer (Fisher Scientific, #22363548) into a fresh 50 mL conical. Cell suspension was centrifuged at 300g for 5 minutes and stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, #P1585, Sigma-Aldrich), 1  $\mu$ M ionomycin (#I9657, Sigma-Aldrich), GolgiSTOP and (#554724, BD Biosciences, 1:1500) GolgiPLUG (#555029, BD Biosciences, 1:1500) in RPMI 1640 medium (#11875119, Life Technologies) containing 10% FBS (#10438026, GIBCO), penicillin/streptomycin (#15140122, GIBCO, 1:100) for 4 h at 37°C in a 5% CO<sub>2</sub> incubator. Following stimulation, T cells were stained with antibodies against surface markers, and thereafter fixed and stained with antibody against intracellular target protein with an intracellular antibody labeling kit following its instructions (#00-5523, eBioscience). Antibodies for flow cytometry were purchased from eBioscience, Biolegend or BD Biosciences and used at a concentration of 1:100. Cells were then analyzed on a Fortessa flow cytometer (BD Biosciences). Treg cells were defined as CD3+CD4+IFN- $\gamma$ -IL-17-IL-10-FOXP3+.

For mCherry/GFP detection in the fecal contents: Content from the specified section of the gut was collected 2 hours after the gavage, homogenized in YPD media (#Y1375, Sigma-Aldrich) and cultured overnight. GFP and mCherry expression was measured by flow cytometry in a Fortessa flow cytometer (BD Biosciences)

#### Instrument

Fortessa Flow Cytometer (BD Bioscience); MACSQuant VYB (Miltenyi Biotec); BD Influx cell sorter (BD Bioscience)

#### Software

BD FACSDIVA; MACSQuantify; BD FACS

#### Cell population abundance

Abundance is reported in figures and methods where relevant.

#### Gating strategy

Gating strategies are specified within the text or figure legend for relevant flow cytometry experiments.

For dose-response assays, yeast were gated from background events by side scatter vs forward scatter (SSC vs FSC)

For cell sorting of yeast, yeast were gated from background events by side scatter vs forward scatter (SSC vs FSC), singlets were then gated from doublets by trigger pulse width vs SSC, and the top ~1% of mCherry expressing cells were then sorted based on FSC vs mCherry signal.

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.