

GPR91 senses extracellular succinate released from inflammatory macrophages and exacerbates rheumatoid arthritis

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When *SUCNR1*/GPR91-expressing macrophages are activated by inflammatory signals, they change their metabolism and accumulate succinate. In this study, we show that during this activation, macrophages release succinate into the extracellular milieu. They simultaneously up-regulate GPR91, which functions as an autocrine and paracrine sensor for extracellular succinate to enhance IL-1 β production. GPR91-deficient mice lack this metabolic sensor and show reduced macrophage activation and production of IL-1 β during antigen-induced arthritis. Succinate is abundant in synovial fluids from rheumatoid arthritis (RA) patients, and these fluids elicit IL-1 β release from macrophages in a GPR91-dependent manner. Together, we reveal a GPR91/succinate-dependent feed-forward loop of macrophage activation and propose GPR91 antagonists as novel therapeutic principles to treat RA.

INTRODUCTION

Macrophages switch their metabolism from oxidative phosphorylation to glycolysis in the presence of oxygen after activation with inflammatory triggers such as TLR-2, -3, -4, and -9 (Rodríguez-Prados et al., 2010). This metabolic switch prepares these cells for rapid activation and synthesis of immune mediators required to perpetuate inflammation (Galván-Peña and O'Neill, 2014). For example, LPS-activated inflammatory (M1) macrophages display a broken Krebs cycle, which ultimately causes intracellular succinate accumulation (Jha et al., 2015). Furthermore, intracellular succinate promotes the stabilization of hypoxia-inducible factor 1 α (HIF-1 α) and enhances proinflammatory IL-1 β production (Tannahill et al., 2013).

The succinate receptor *SUCNR1*/GPR91 is a G protein-coupled cell surface sensor for extracellular succinate (He et al., 2004). Its activity has been linked to metabolic indications such as diabetic retinopathy (Toma et al., 2008), diabetic renal disease (Peti-Peterdi et al., 2008), hypertension (He et al., 2004; Sadagopan et al., 2007), and atherothrombosis (Macaulay et al., 2007).

Within the immune system, GPR91 is expressed on immature DCs and macrophages (Rubic et al., 2008). Activation

of DCs via GPR91 favors their maturation and migration to lymph nodes, augments TLR-induced proinflammatory cytokine production, and enhances antigen-specific activation of T helper cells (Rubic et al., 2008). Thus, extracellular succinate acts as an alarmin by locally activating GPR91-expressing immature DCs to initiate or exacerbate immune responses.

It has been postulated that succinate reaches the extracellular milieu after necrosis or pathological damage of inflamed tissue (Rubic et al., 2008). Indeed, extracellular succinate increases as a consequence of ischemia or hypoxia (Ariza et al., 2012), with local tissue levels reaching millimolar concentrations in the respective rodent models (Correa et al., 2007; Toma et al., 2008; Chouchani et al., 2014). In humans, succinate has been abundantly detected in synovial fluid (SF) from rheumatoid arthritis (RA) patients (Borenstein et al., 1982; Kim et al., 2014), and a metabolic profiling study has identified succinate as the most differentially expressed metabolite in RA compared with other arthropathies (Kim et al., 2014).

Sustained proinflammatory activity of glycolytic macrophages is central in RA (McInnes and Schett, 2007). The number of synovial CD68⁺ macrophages correlates with disease severity (Haringman et al., 2005), and their reduction correlates with clinical response to therapy (Bresnihan et al., 2009). We hypothesized that macrophage activation in RA will

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Abbreviations used: AIA, antigen-induced arthritis; AUC, area under the curve; HIF-1 α , hypoxia-inducible factor 1 α ; IC, ion chromatography; mBSA, methylated BSA; M-CSF, macrophage CSF; MS, mass spectrometry; MSU, monosodium urate; NGS, normal goat serum; NIR, near infrared; RA, rheumatoid arthritis; SF, synovial fluid.

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result in accumulation of succinate in the interstitial environment, thus fueling tissue inflammation via GPR91 activation.

RESULTS AND DISCUSSION

To study how extracellular succinate and GPR91 regulate macrophage function, we generated macrophages *in vitro* by differentiation of mouse WT or *Sucnr1*-deficient (*Sucnr1*^{-/-}) BM cells under neutral (macrophage CSF [M-CSF]) or inflammatory (M-CSF + IFN- γ) conditions. We confirmed that macrophages from both sources and exposed to the different conditions differentiated normally *in vitro* by assessing their expression of F4/80, inducible nitric oxide synthase, and TLR4 (unpublished data). Activation of WT BMDMs with LPS or IL-1 β induced a 10- and 3-fold increase of *Sucnr1* mRNA, respectively, suggesting that inflammation may prepare macrophages for extracellular succinate sensing (Fig. 1 A). *Sucnr1* expression mediated by IL-1 β but not by LPS was reduced by inhibitors of I κ B and p38 kinases, implying a direct effect of this cytokine and of additional TLR4-driven mediators in the induction of GPR91 (unpublished data). Because inflammatory conditions such as RA lead to accumulation of extracellular succinate (Kim et al., 2014), we asked whether inflammatory triggers promote the secretion of succinate from macrophages to the extracellular space. Activation of neutral or inflammatory BMDMs *in vitro* with LPS led to a significant release of succinate into the culture medium (Fig. 1 B). This effect was independent of GPR91 expression, and strikingly, extracellular succinate was found more abundantly in cultures of *Sucnr1*^{-/-} BMDMs than in corresponding WT cells (Fig. 1 B). Succinate release appears to be a specific function of activated macrophages (Ariza et al., 2012) rather than the consequence of macrophage death, as we observed no differences in lactate dehydrogenase release or expression of cell death markers upon LPS stimulation (unpublished data). Consequently, we asked whether the inflammatory responses of BMDMs could be affected by extracellular succinate. We differentiated neutral and inflammatory BMDMs and found that in the WT cells, LPS stimulated IL-1 β release over basal conditions. Furthermore, WT inflammatory macrophages produced four times higher levels of IL-1 β than WT BMDMs differentiated under neutral conditions. More notably, we observed that LPS-activated BMDMs from *Sucnr1*^{-/-} mice showed a profound decrease of IL-1 β release, IL-1 β mRNA, and pro-IL-1 β production compared with their WT controls (Fig. 1, C and D; and not depicted). We also found that *Sucnr1*^{-/-} inflammatory macrophages were deficient in their IL-1 β release upon activation with the classical inflammasome stimulus LPS/monosodium urate (MSU; Fig. 1 E).

Increased intracellular succinate has been shown to promote IL-1 β transcription via stabilization of HIF-1 α after LPS stimulation (Tannahill et al., 2013). Therefore, we tested whether GPR91 activation by extracellular succinate impacts HIF-1 α stability. Extracellular succinate alone led to a moderate induction of HIF-1 α in inflammatory WT BMDMs

and significantly enhanced LPS-induced HIF-1 α expression (Fig. 1 F). Conversely, although LPS induced a robust HIF-1 α signal in *Sucnr1*^{-/-} BMDMs, there was no enhancing effect by exogenous addition of succinate or by the abundant levels present in the cultures after LPS stimulation (Fig. 1 B). These data suggest that in an inflammatory environment, macrophages recycle extracellular succinate via GPR91 to up-regulate a HIF-1 α -dependent innate pathway, which ultimately potentiates IL-1 β production.

To understand whether extracellular succinate modified macrophage responses *in vivo*, we chose to study arthritis in mice. Our rationale was based on the fact that extracellular succinate accumulates in the SF of RA patients (Kim et al., 2014) and that RA is a disease with strong macrophage involvement (Richards et al., 1999). In analogy to the human situation, we detected high concentrations of succinate in SF from mice with antigen-induced arthritis (AIA) compared with SFs from naive mice (Fig. 2 A). Next, we investigated the functional role of GPR91 in this model. We found that GPR91 deficiency led to a significant reduction ($48.4 \pm 4\%$) of knee swelling (Fig. 2 B). Analysis of reciprocal BM chimeras indicated that irradiated WT recipients transfused with *Sucnr1*^{-/-} BM cells were protected to the same degree as *Sucnr1*^{-/-} animals transfused with *Sucnr1*^{-/-} BM (Fig. 2 C). This suggested that the disease-enhancing effect of extracellular succinate is mediated by GPR91-expressing hematopoietic cells in the AIA model.

Because synovial macrophages are involved in the development of AIA (Richards et al., 1999) and these cells express GPR91 (Rubic et al., 2008), we tested whether macrophages were the key GPR91-expressing cell type driving arthritis in this model. To this end, we combined the AIA model with a near-infrared (NIR) probe conjugated to folate to visualize activated folate receptor B-expressing macrophages within inflamed joints (Kelderhouse et al., 2015). On day 2 of arthritis development, i.e., when swelling was maximal, the probe was injected *i.v.*, and signals were monitored over the next 72 h. A strong signal for activated macrophages was specifically observed in the knees of WT mice treated with antigen over the entire observation period. However, the intensity of the reporter was significantly reduced in *Sucnr1*^{-/-} mice compared with WT counterparts (Fig. 2 D). These data suggested that macrophages sense extracellular succinate within inflamed joints via GPR91, thus contributing to their activation, and that this mechanism was a central driver of arthritis. To discount the possibility that the observed differences were caused by overall reduced monocyte/macrophage numbers in *Sucnr1*^{-/-} mice, we enumerated monocytes and macrophages in spleen and BM of healthy WT and *Sucnr1*^{-/-} mice and found similar numbers in both strains (unpublished data).

Next, we addressed the molecular mechanism of how intraarticular GPR91-activated macrophages contributed to arthritis *in vivo*. Immunohistochemical staining of the joints of WT mice on day 7 of AIA revealed that activated macrophages (e.g., of the synovial lining or within the synovium)

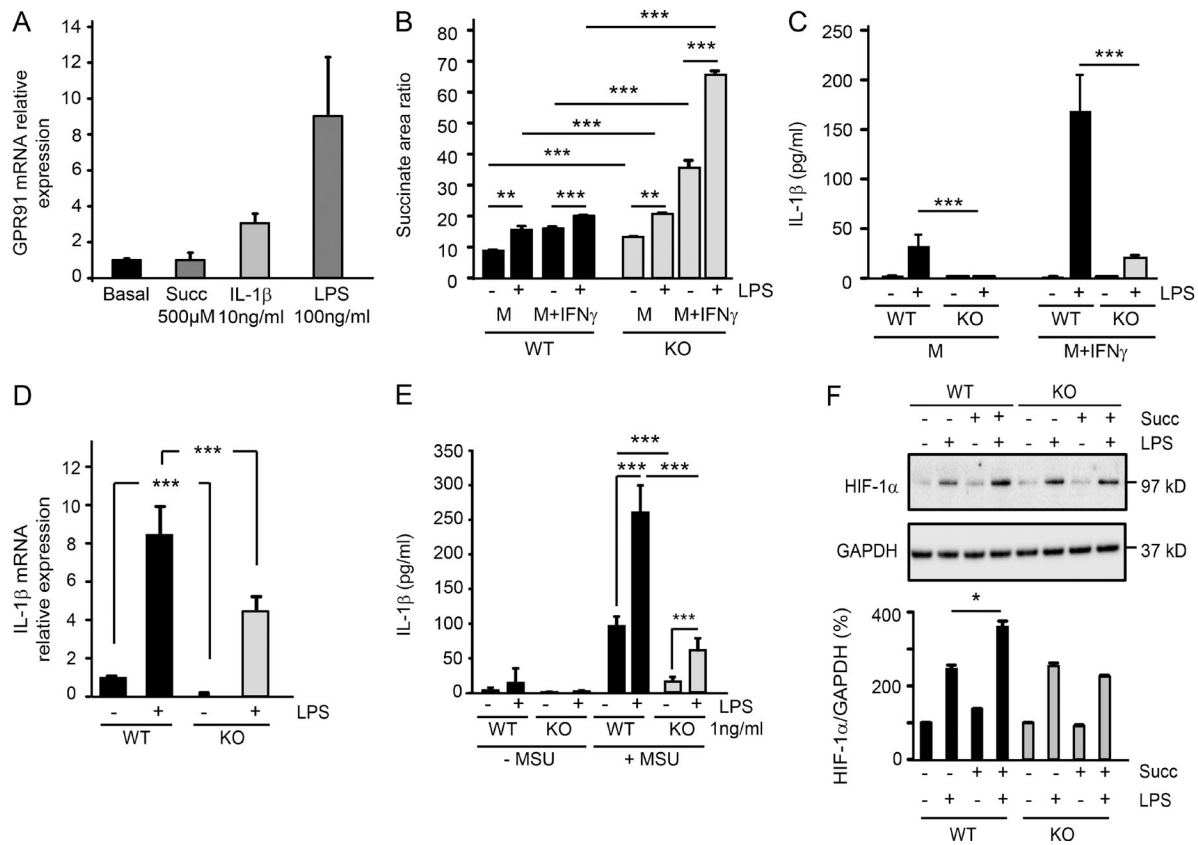


Figure 1. Extracellular succinate signals via GPR91 to stimulate macrophages to release IL-1 β . (A) GPR91 mRNA expression in WT (Janvier C57BL/6J) inflammatory BMDMs (M-CSF + IFN- γ) \pm 100 ng/ml LPS, 500 μ M succinate, or 10 ng/ml IL-1 β for 24 h. $n = 3$ of Ct values. Succinate (Succ), IL-1 β , and LPS related to basal (=1). Data are representative of three experiments. (B) Succinate levels (mass spectrophotometry area ratio) in medium from cultured BMDMs. Extracellular succinate from WT (littermates; black bars) and *Sucnr1*^{-/-} (gray bars), neutral (M, M-CSF), or inflammatory (M + IFN- γ) BMDMs \pm 100 ng/ml LPS for 24 h is shown. $n = 6$ wells. Data are representative of three experiments. (C) IL-1 β in supernatants of WT (Janvier C57BL/6J) and *Sucnr1*^{-/-} neutral or inflammatory BMDMs \pm 100 ng/ml LPS for 24 h. $n = 3$ wells and are representative of seven experiments. (D) IL-1 β mRNA levels from cell lysates from WT (Janvier C57BL/6J) or *Sucnr1*^{-/-} inflammatory BMDMs \pm 100 ng/ml LPS at 4 h (related to WT basal = 1). $n = 2$ –3 of Ct values. Data are representative of two experiments. (E) IL-1 β levels measured in the supernatant of WT (Janvier C57BL/6J) and *Sucnr1*^{-/-} inflammatory BMDMs stimulated with 1 ng/ml LPS and 180 μ g/ml MSU. $n = 5$ –6 wells. Data are representative of two experiments. (F) Western blot of HIF-1 α (representative blot of two experiments) and quantification (two experiments; 100% for no stimulus, WT, and *Sucnr1*^{-/-}) at 6 h after stimulation with 500 μ M succinate, 100 ng/ml LPS, or a combination of the two in WT littermate controls and *Sucnr1*^{-/-} inflammatory BMDMs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, unpaired Student's t test. Data are means \pm SEM.

were potent IL-1 β producers (Fig. 2 E). Consistent with this observation, IL-1 β expression in synovial knee tissue of *Sucnr1*^{-/-} mice undergoing AIA was significantly lower than in WT counterparts (Fig. 2 F). Expression of other inflammatory mediators (TNF, IL-6, IL-12p35, IL-23p19, and CCL2) was somewhat reduced in *Sucnr1*^{-/-} mice compared with WT mice; yet, these differences did not reach significance (not depicted). This observation is in line with literature showing that IL-1 β is the dominant cytokine in AIA joints at this time of disease (Simon et al., 2001). Our data indicate that elevated extracellular succinate levels within the SF of arthritic joints activate macrophages via GPR91 to up-regulate IL-1 β production and exacerbate arthritis.

Finally, we aimed to analyze the role of GPR91 activation for IL-1 β release in the context of human RA. For

this purpose, we incubated human RA SFs with BMDMs from WT and *Sucnr1*^{-/-} mice. Fluids from RA patients elicited greater IL-1 β production from WT mouse macrophages than from corresponding *Sucnr1*^{-/-} cells. The extent of the response correlated with the amount of succinate present in the SF (Fig. 3 A). Furthermore, we observed that succinate levels in the SF of collagen-induced arthritis mice correlated with paw swelling in vivo (Fig. 3 B), indicating that succinate levels could be used as a biomarker of disease severity. Human myeloid U937 cells constitutively express GPR91 (unpublished data) and mobilize calcium fluxes in response to succinate (mean EC₅₀, 96 μ M). Using these cells, we found that the succinate content in human RA SF correlated with their ability to release IL-1 β (Fig. 3 C). To further demonstrate that succinate in rheumatoid SFs acted directly via GPR91,

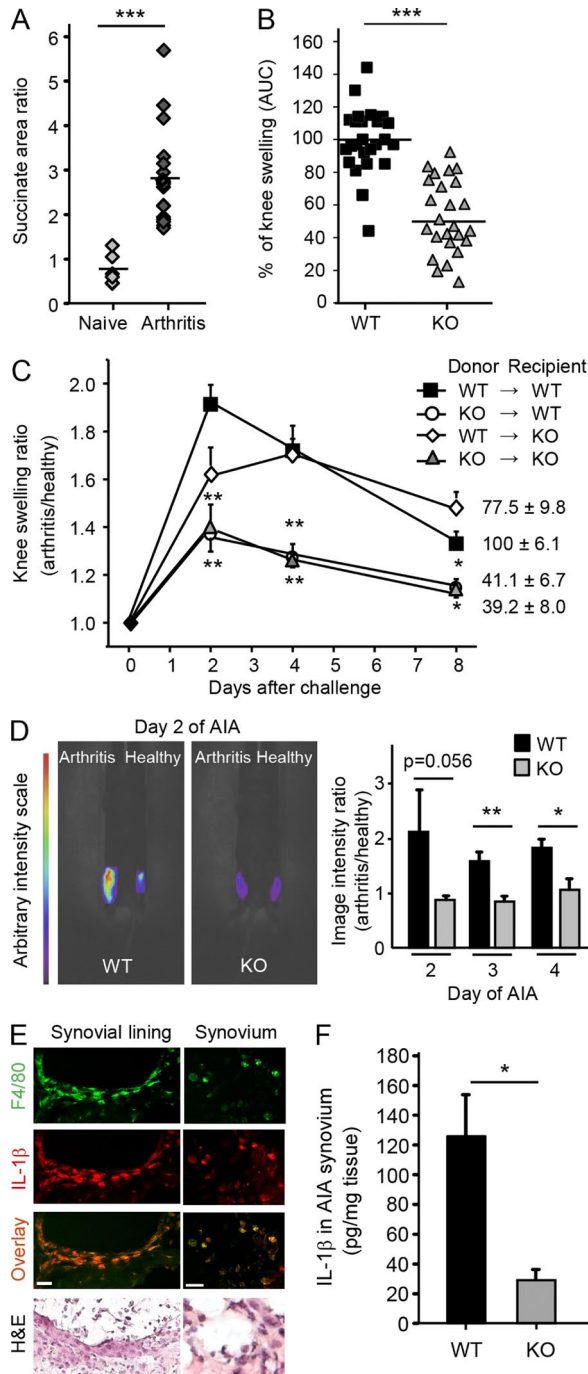


Figure 2. Extracellular succinate levels within the SF of arthritic joints activate macrophages via GPR91 to up-regulate IL-1 β production. (A) Succinate area ratio (mass spectrophotometry) in SF from knees of Janvier C57BL/6J mice on day 7–8 of AIA ($n = 19$; black diamonds) versus naive controls ($n = 5$; gray diamonds) pooled from five independent experiments. The lines depict means. A Mann-Whitney rank sum test was used, as the normality test failed (Shapiro-Wilk). (B) AUCs of knee swelling ratio curves (arthritis/healthy) over time ($n = 25$ per genotype), pooled from five experiments. The lines depict means. For each experiment, the WT (both Janvier C57BL/6J and littermate controls) mice were accorded a mean of 100%. WT (black squares) and *Sucnr1*^{-/-} (gray triangles) were compared

we used the GPR91 antagonist GPR91A1 (compound 4c in Bhuniya et al., 2011). GPR91A1 blocked IL-1 β release from U937 cells activated with human SF or exogenous succinate (Fig. 3 D). Unfortunately, the weak cross-reactivity of GPR91A1 to rodent GPR91 and its low oral bioavailability precluded its *in vivo* use.

Our results position GPR91 antagonists as an attractive therapeutic option to interfere with the local pathogenic process occurring in arthritic joints upstream of IL-1 β and possibly of other inflammatory mediators. This therapeutic concept is further supported by the correlation of synovial macrophage numbers with disease severity (Haringman et al., 2005; Bresnihan et al., 2009) and the clinical efficacy of IL-1 β -neutralizing antibodies or the recombinant human IL-1R antagonist in juvenile idiopathic arthritis and RA, respectively (Dinarello and van der Meer, 2013; Rondeau et al., 2015). However, the therapeutic effect of GPR91 antagonists in arthritis will likely go beyond reduction of succinate-triggered IL-1 β release, as GPR91 activation stimulates other nonimmune and immune cells; e.g., in DCs, it promotes antigen presentation and chemotaxis and amplifies TLR-mediated cytokine release (Rubic et al., 2008).

The SF of RA patients contains various endogenous TLR ligands that can act as triggers for innate and adaptive immune processes (Kennedy et al., 2011). We found that activation of macrophages with many TLR ligands leads to succinate release into the extracellular space, potentially facilitating GPR91-dependent succinate sensing and subsequent augmentation of IL-1 β production (unpublished data). Therefore, our observation may not be restricted to TLR4 activation, and the effect could extend to diverse settings of macrophage activation.

On a more general level, our data unravel a metabolic mechanism used by macrophages to alert neighboring cells in situations of tissue inflammation. By switching from oxidative phosphorylation to glycolysis, macrophages change

by unpaired Student's *t* test. (C) Knee swelling ratio (arthritis/healthy) over time in reciprocal BM chimeras of WT (littermates or congenic CD45.1 SJL-Ptprca/BoyAiTac; Taconic) and *Sucnr1*^{-/-} mice. (Right) AUC (percentage) expressed as means \pm SEM. $n = 5$ per group and $n = 4$ in the KO-KO group. One-way ANOVA and Tukey's posttest were used. The experiment was performed once. (D, left) NIR intensity images of folate-positively activated macrophages in knees of WT littermates and *Sucnr1*^{-/-} animals on day 2 of AIA. $n = 10$ per group. (Right) Quantification of the NIR signal in knees of WT and *Sucnr1*^{-/-} animals over 72 h after probe injection. Bars show means of NIR intensity ratios in knees (arthritis/healthy) \pm SEM. $n = 10$ per bar, unpaired Student's *t* test. Data are representative of two experiments. (E) Colocalization of F4/80-positive macrophages (green) with IL-1 β (red) from the synovial lining and synovium of WT mice (littermates). Hematoxylin and eosin (H&E) staining shows tissue morphology. Bars, 25 μ m. Images are representative of 30 sections from five mice. (F) IL-1 β levels in synovial tissue homogenates of WT littermate or *Sucnr1*^{-/-} mice on day 7 of AIA. Data are representative of two experiments. Error bars represent means \pm SEM of five mice per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, unpaired Student's *t* test.

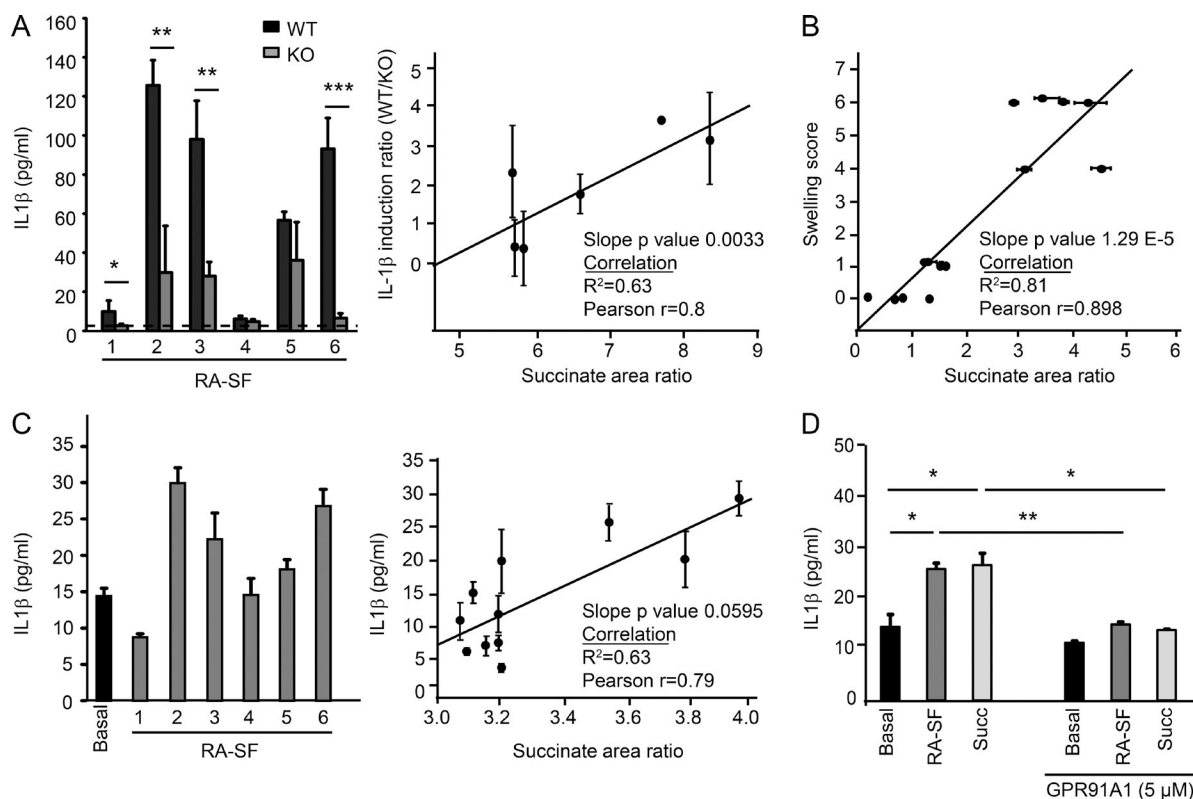


Figure 3. IL-1 β release is suppressed in *Sucnr1*^{-/-} or GPR91 antagonist-treated human macrophages incubated with RA SF. (A, left) IL-1 β production from WT (littermates) and *Sucnr1*^{-/-} (gray) BMDMs incubated for 24 h with 10% human RA SF from six patients (RA SF 1–6). Error bars represent means \pm SEM of triplicates. Data are representative of two experiments (unpaired Student's *t* test). Where normality failed (Shapiro-Wilk), a Mann-Whitney rank sum test was used. The dashed line is the limit of detection. (Right) IL-1 β induction ratios (from WT littermate/*Sucnr1*^{-/-} BMDMs) by the six RA SFs were correlated with concentration of succinate in the SFs. Means \pm range of the induction ratio for each SF from the two experiments (Pearson's correlation) are shown. (B) Correlation of paw swelling with SF succinate concentrations within the same paws, measured in duplicates (collagen-induced arthritis, DBA/1; *n* = 13; Janvier). Pearson's correlation was used. (C, left) IL-1 β production from U937 cells incubated for 24 h with 10% RA SF from six RA patients (gray bars, RA-SF 1–6) or medium only (basal). Data are means \pm SEM of triplicates and representative of two experiments. (Right) The amount of IL-1 β elicited by 11 RA SFs was correlated with the concentration of succinate in the SF. Means of IL-1 β \pm the range for each SF from the two experiments (Pearson's correlation) are shown. (D) IL-1 β production from U937 cells incubated for 24 h with 10% RA SF or 1 mM succinate (Succ) in the presence or absence of 5 μ M GPR91 antagonist GPR91A1. Error bars represent means \pm SEM of triplicates. Data are representative of five experiments. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001, unpaired Student's *t* test.

their energy metabolism (Jha et al., 2015). We now provide an example of how energy metabolites may feed back on an immune phenotype of activated macrophages as proposed in a recent review (O'Neill and Pearce, 2016). The accumulation of extracellular succinate as a consequence of this metabolic switch leads to GPR91-dependent recycling of succinate, which results in increased IL-1 β . This mechanism fuels inflammation in an autocrine manner and propagates inflammation by alerting neighboring cells of the immunological danger (Fig. 4). Therefore, GPR91-driven recycling of extracellular succinate by macrophages is one example of how the immune system utilizes excess metabolites that are not used for its primary purpose, the generation of metabolic energy. This extends the concept of immunometabolism, as it shows how an extracellular metabolite is recycled to amplify inflammatory macrophage responses.

MATERIALS AND METHODS

Human samples and mice

Human SF samples were obtained from Asterand under patient informed consent, protocol number AST-CP-005. Activities of Asterand and their collaborators were conducted in accordance with applicable laws, regulations, and ordinances. The study protocol was approved by the Novartis Research Center ethical committee and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

All mice were used in accordance with Swiss Federal and Cantonal Authorities. *Sucnr1*^{-/-} mice on a C57BL/6 background were generated by Deltagen by replacement of part of exon 2 (5'-GGCTACCTCTTCTGCAT-3') with a lacZ-neomycin cassette as described previously (Rubic et al., 2008). Many transgenic mice harbor an *Rd8* mutation in the *Crb1* gene because of the C57BL/6N origin of the embry-

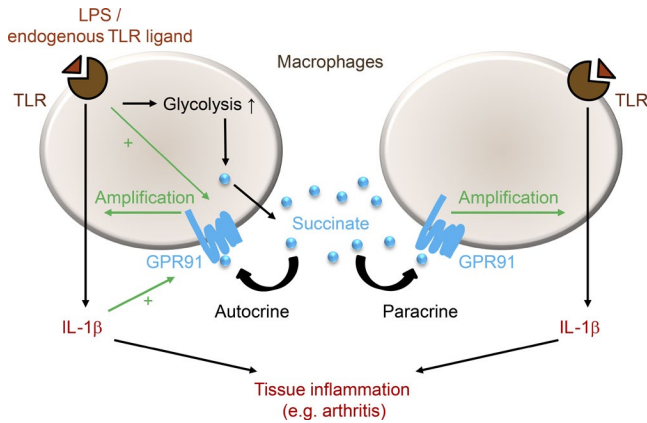


Figure 4. Proposed mechanism of GPR91-driven autocrine and paracrine enhancement of IL-1 β release from activated macrophages. Endogenous TLR ligands in the SF of RA patients activate macrophages locally. This leads to an enhancement of glycolysis and an increase of intracellular succinate. At the same time, succinate is released to the extracellular milieu where it binds to GPR91 and amplifies IL-1 β production from either the same or a neighboring GPR91-expressing cell. Both LPS and IL-1 β up-regulate GPR91 in a further feed-forward action to perpetuate inflammation.

onic stem cells (Mattapallil et al., 2012). For this reason, and to eliminate potential artifacts, we extensively backcrossed (10 generations) *Sucnr1*^{-/-} KO mice onto a C57BL/6/J background where the mutation is absent. WT mice were either littermates or purchased from Janvier (C57BL/6J), as indicated. The results were not different between the WT mice from the different origins. Mice were used between 7 and 12 wk of age in age-matched groups. For reciprocal BM chimeras, to assess the level of chimerism (always >90% at 8–10 wk), congenic CD45.1 mice (SJL-Ptprca/BoyAiTac;Taconic) were used as WT recipients and as donors in the WT \rightarrow *Sucnr1*^{-/-} group. All other mice used in this experiment were WT and *Sucnr1*^{-/-} littermates. To ascertain succinate levels in collagen-induced arthritis swollen joints, DBA/1 mice (Janvier) were used.

AIA

Mice were sensitized intradermally on the back at two sites to methylated BSA (mBSA) homogenized 1:1 with complete Freund's adjuvant on days 21 and 14 (0.1 ml containing 1 mg/ml mBSA). On day 0, the right knee was injected with 10 μ l of 10 mg/ml mBSA in 5% glucose solution (antigen-injected knee), and the left knee was injected with 10 μ l of 5% glucose solution (vehicle-injected knee). The diameters of the left and right knees were measured using calipers immediately after the intraarticular injections (day 0) and again on days 2, 4, and 7 or 8.

Knee swelling ratios were calculated as right/left knee swelling and plotted over time. Areas under the curve (AUCs) for control and treatment groups were derived from these curves. The percentage of inhibition of the treatment group AUC was calculated versus the control group AUC (0% inhibition) using Excel.

For IL-1 β measurements in the synovial tissue, the synovial tissue was extracted from exposed knee joints and homogenized with a Polytron homogenizer (D-9; Micra) in cell lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail set I (EMD Millipore). After protein quantification with a bicinchoninic acid kit (Thermo Fisher Scientific), samples were subjected to a Bioplex assay (Bio-Rad Laboratories) according to the manufacturer's instructions.

For folate imaging, the Cy5.5-folate probe was synthesized in house in two steps starting from folic acid (Sigma-Aldrich) as previously described (Tung et al., 2002). At 3 h after intraarticular knee challenge (day 0), 1 mg of a 10 ml/kg solution of Cy5.5-folate in PBS was injected i.v. via the tail vein. Spectral fluorescence images were obtained using an in vivo imaging system (Maestro; CRi Inc.). A band-pass filter (Cy5.5; peak excitation at 675 nm; peak emission at 694 nm; long-pass filter; acquisition settings at 640–820) was used for emission and excitation light. The tunable filter was stepped automatically in 10-nm increments, whereas the camera-captured images were at an automatic exposure.

To evaluate signal intensities, regions of interest were selected over the knee areas, and the mean fluorescence signal (expressed as photons/squared centimeters) from those areas was determined. Data were expressed as a ratio between right and left signals. The spectral fluorescent images consisting of autofluorescence spectra and Cy5.5 dye were captured and unmixed on the basis of their spectral patterns using the Maestro software (CRi Inc.).

BM irradiation chimera

8-wk-old female C57Bl6 WT littermate or SJL-Ptprca/BoyAiTac (CD45.1 congenic; Taconic) mice and *Sucnr1*^{-/-} mice were irradiated with 4.5 Gy and then again 5 h later with 4.5 Gy. 30 min after the second irradiation, 6.1×10^6 BM cells from donor mice (WT littermate, SJL-Ptprca/BoyAiTac, or *Sucnr1*^{-/-} mice) were injected into the irradiated mice i.v. via the tail vein. Animals were monitored over the following 8–10 wk for recovery and engraftment (>90% chimerism) before being subjected to AIA (see the previous section).

Immunohistochemistry

Frozen sections of synovial tissue (10 μ m) were blocked with 10% normal goat serum (NGS) in PBS/0.1% Triton X-100 for 30 min at room temperature. Rabbit anti-IL-1 β (1:100; Abcam) was added in 3% NGS in PBS/0.1% Triton X-100 overnight at 4°C. After three washes in PBS, the secondary goat anti-rabbit Alexa Fluor 546 (1:200; Molecular Probes) and primary rat anti-mouse F4/80-FITC (1:50; Abcam) were added for 2 h at room temperature in 3% NGS in PBS/0.1% Triton X-100. After three washes in PBS, slides were mounted with ProLong Gold Antifade reagent (Invitrogen). Staining of sections with the secondary antibody alone did not show background staining, and staining of the secondary antibody together with F4/80-FITC did not reveal cross-reactivity of the secondary antibody with the F4/80 antibody. Images

were acquired on a microscope (DM6000B) with a camera (DFC360; Leica Biosystems) and analyzed with the Leica Application Suite software.

Generation of mouse macrophages

Mouse BMDMs were obtained by flushing the femurs, humeri, and tibiae of WT (littermates and Janvier C57BL/6J) and *Sucnr1*^{-/-} mice with PBS. Monocytes were isolated using the Easysep Mouse Monocyte Enrichment kit (STEMCELL Technologies) and cultured for 6 d in RPMI-1640 medium supplemented with 10% FCS (PAA Laboratories), 1% penicillin and streptomycin, 1% MEM nonessential amino acids, 50 μ M β -mercaptoethanol, 1 mM sodium pyruvate, and 25 mM Hepes (all supplements from Gibco). 40 ng/ml recombinant mouse M-CSF was added on day 0, and for inflammatory macrophages, 50 ng/ml mouse recombinant IFN- γ was added for the last 2 d (both from R&D Systems).

In vitro stimulation of mouse macrophages or U937 cells

U937 or mouse macrophages derived from 7–9-wk-old age-matched animals were incubated for up to 24 h in 96-well flat-bottom plates at a density of 10^5 cells per well in RPMI-1640 containing 1 or 100 ng/ml LPS (InvivoGen), 500 μ M succinate (sodium succinate dibasic hexahydrate; Sigma-Aldrich), 10 ng/ml mouse IL-1 β (PeproTech), 180 μ g/ml MSU (Enzo Life Sciences), 5 μ M GPR91 antagonist GPR91A1 (synthesized in house from Bhuniya et al., 2011), and 10% human RA SFs (Asterand). After 24 h (or 7 h in the case of culture with 1 ng/ml LPS [2 h] followed by 180 μ g/ml MSU [5 h]), cells were collected and processed for quantitative PCR as described in the next section. Cytokines in supernatants were analyzed by ELISA using the mouse IL-1 β ELISA Set or human IL-1 β ELISA Set II (both from BD) according to the manufacturer's instructions.

Quantitative PCR

Total RNA was extracted from cells using the RNeasy Mini kit (QIAGEN). cDNA was prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), and a SimpliAmp thermal cycler and the Quant Studio 12K Flex system (Applied Biosystems) were used for quantitative PCR. The results are presented as relative quantification versus the basal condition using the comparative Ct method.

For quantification of mouse GPR91 and human EF-1 α , primers and probes were designed and purchased from Microsynth (mGPR91 forward [5'-TCACTGTGGTGT TTGGCTACCT-3'], reverse [5'-CCCTTATCATTGGCA TAACTCTTTATC-3'], and probe [5'-TTTGCTTTCCTG TGCACCCTTCCCAT-3']; hEF-1 α forward [5'-TTTGAG ACCAGCAAGTACTATGTGACT-3'], reverse [5'-TCA GCCTGAGATGTCCCTGTAA-3'], and probe [5'-TCA TTGATGCCCCAGGACACAGAGAC-3']). Expression of all other genes was measured using Taqman gene assays kits (Applied Biosystems): human GPR91 (Hs00263701-m1), mouse IL-1 β (Mm00434228_m1), mouse β -glucuronidase

(Mm00446953_m1), mouse inducible nitric oxide synthase (Mm00440502_m1), mouse F4/80 (Mm00802529_m1), and mouse TLR4 (Mm00445273_m1).

Western blotting

Lysates from inflammatory BMDMs stimulated for 6 h with 500 μ M succinate, 100 ng/ml LPS, or a combination were subjected to Western blotting according to standard protocols. The antibodies used were rabbit anti-HIF-1 α (1:1,000; Novus Biologicals) and rabbit anti-GAPDH (1:5,000; Cell Signaling Technology). Secondary antibody was goat anti-rabbit IgG and HRP conjugate (Bio-Rad Laboratories). Bands were quantified on the Chemi DocMP imaging system (Bio-Rad Laboratories) using the Image Lab software (version 5.2.1 for PC; Bio-Rad Laboratories). For quantification, the HIF-1 α band intensity was divided by the GAPDH intensity. The basal, unstimulated WT and *Sucnr1*^{-/-} BMDMs were accorded 100%, and the stimulated conditions were compared with the basal conditions.

Ion chromatography (IC)-tandem mass spectrometry (MS [IC-MS/MS]) analysis of succinate

For assessment of succinate by MS, 18.2 M Ω Milli-Q water was used for the eluent, and regeneration of the IC system was generated by a PureLab Ultra water purification system (Elga-Veolia). Methanol (Optima LC/MS grade) was purchased from Thermo Fisher Scientific. The succinic acid and the internal standard [¹³C₄]succinic acid were purchased from Sigma-Aldrich.

1 μ l SF was extracted with 900 μ l of 55 nM internal standard solution in 90% methanol. The supernatant was evaporated under nitrogen at 40°C, and the residues were redissolved in 100 μ l Milli-Q water. 2 μ l was injected for IC-MS/MS analysis. 200 μ l of cell culture medium was extracted with 600 μ l of 90 nM internal standard solution in 90% methanol. The supernatant was evaporated under nitrogen at 40°C, and the residues were redissolved in 200 μ l Milli-Q water. 0.4 μ l was injected for IC-MS/MS analysis.

An IC system (ICS-5000+; Thermo Fisher Scientific) was coupled to a mass spectrometer (QTrap5500; Sciex) with electrospray ionization. The chromatographic separation was achieved using an analytical column (4 μ m, 0.4 \times 150 mm; IonPac AS18; Dionex) and a guard column (4 μ m, 0.4 \times 35 mm; IonPac AG19; Dionex) at a flow rate of 17 μ l/min and at 35°C. The potassium hydroxide gradient program was 0 min at 10 mM, 1 min at 33 mM, 13.5 min at 36 mM, 23.7 min at 117.6 mM, 28.1 min at 117.6 mM, 28.2 min at 10 mM, and 32 min at 10 mM. The suppressor current was set at 25 mA, and the suppressor was operated in external water mode with a regenerate flow rate of 19 μ l/min. A make-up flow of 9 μ l/min of methanol was combined with the IC flow before MS to enhance the ionization. The MS was operated in negative ion mode with multiple reaction monitoring. Statistics, e.g., Pearson correlation and Student's *t* test, were done using Genedata Analyst 9.0 (Genedata AG).

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