GPR91 deficiency exacerbates allergic contact dermatitis while reducing arthritic disease in mice

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Abstract

Background: Succinate, in addition to its role as an intermediary of the citric acid cycle, acts as an alarmin, initiating and propagating danger signals resulting from tissue injury or inflammatory stimuli. The contribution of this immune sensing pathway to the development of allergic and inflammatory responses is unknown.

Methods: Ear thickness of wild-type (wt) and Sucnr1-deficient (Sucnr1−/−) mice, sensitized and challenged with oxazolone, was used as a criterion to assess the relevance of SUCNR1/GPR91 expression mediating allergic contact dermatitis (ACD). Results obtained in this system were contrasted with data generated using passive cutaneous anaphylaxis, ovalbumin-induced asthma and arthritis models.

Results: We found augmented ACD reactions in Sucnr1−/− mice. This observation correlated with increased mast cell activation in vitro and in vivo. However, exacerbated mast cell activation in Sucnr1−/− mice did not contribute to the enhancement of asthma or arthritis and seemed to be due to alterations during mast cell development as augmented mast cell responses could be recapitulated in wt mast cells differentiated in the absence of succinate.

Conclusions: A deficiency in succinate sensing during mast cell development confers these cells with a hyperactive phenotype. Such a phenomenon does not translate into exacerbation of asthma or mast cell-dependent arthritis. On the contrary, the fact that Sucnr1−/− mice developed reduced arthritic disease, using two different in vivo models, indicates that GPR91 antagonists may have therapeutic potential for the treatment of allergic and autoimmune diseases.

Succinate is an intermediate of the citric acid cycle, which is generated in mitochondria following hydrolytic release of CoA from succinyl-CoA. Succinate can accumulate in mitochondria and get exported to the extracellular milieu when there is an imbalance between nutrients and/or oxygen and energy demands (1). Extracellular succinate binds to its specific receptor, SUCNR1/GPR91 (2), on the plasma membrane of hepatic, renal, retinal and immune cells, instructing them to secrete hormones, growth factors or cytokines involved in multiple normal and pathologic processes (3). There is evidence indicating that triggering GPR91 prevents premature subretinal age-related macular degeneration-like lesions (4) and facilitates the recovery from cerebral hypoxic-ischemic events (5). On the other hand, the sensory function of GPR91 detecting succinate following ischemic stress has been implicated in the mechanisms governing diabetic retinopathy (6, 7), causing cardiac hypertrophy (8, 9) and eliciting hepatic stellate cell activation (10) and hepatic fibrosis (11). In addition to hypoxia, elevated glucose levels, as observed in diabetes, also result in the export of succinate to the extracellular environment (12, 13). In this case, increased interstitial levels of succinate are also sensed by GPR91 in the kidney juxtapaglomerular apparatus (14) and macula densa cells (15), causing the release of renin, which ultimately lead to hypertension and renal tissue injury (13). Furthermore, our laboratory has previously shown that succinate can be released as a consequence of necrosis following tissue damage. We demonstrated that immature dendritic cells (DC) express GPR91 and utilize this receptor to sense immunologic danger and to enhance antigen-presenting functions required for optimal T-cell
activation (16). We hypothesized that GPR91 plays a broader role in immune regulation, and we therefore aimed to examine its pathophysiologic or protective role in the context of allergic and inflammatory challenges.

**Methods**

**Animals**

Sucnr1-deficient (Sucnr1<sup>−/−</sup>) mice were generated as previously described (16). The original model was generated in the C57BL/6N background. These mice featured the single-nucleotide deletion in the Crbl gene reported by Mattapallil et al. (17), which is present in all C57BL/6N substrains and can lead to ocular phenotypes. Thus, Sucnr1<sup>−/−</sup> mice were backcrossed (>10 generations) onto the C57BL/6J background (Janvier Labs, Le Genest-Saint-Isaule, France), which does not carry this mutation. The immune phenotype and functions of the Sucnr1<sup>−/−</sup> mice on C57BL/6J and C57BL/6J substrains were equivalent. Furthermore, the immune phenotype of Sucnr1<sup>−/−</sup> mice was normal and comparable to that of wild-type (wt) mice (Fig. S1). All experiments were performed using 8- to 16-week-old mice. All animal studies described in this report were performed according to Swiss animal protection laws issued by the Cantonal Veterinary Office Basel-Stadt, Switzerland, by the Austrian Animal Experimentation Law and by the Novartis Animal Welfare Policy.

**Animal models**

Allergic and inflammatory responses were conducted in parallel on wt and Sucnr1<sup>−/−</sup> mice. Elicitation and sensitization for allergic contact dermatitis (ACD) was performed with 1% oxazolone (Sigma-Aldrich, Buchs, Switzerland) in acetone as previously described (18). RNA was extracted from ear tissue and subjected to real-time RT-PCR analysis for mouse EF-1α, IL-1β, IL-4, IL-5, IL-13, IL-17, IFN-γ and TNF-α. Irritant contact dermatitis (ICD) was induced by a single topical application of 10 μl of 0.005% 12-O-tetradecanoylphorbol-13-acetate (TPA) (wt/vol in acetone; Sigma-Aldrich) on the inner and outer surfaces of the left ears of wt and Sucnr1<sup>−/−</sup> mice. The right ears were treated with an acetone mixture to correct for fluctuations in signal strength during acquisition time. Data analysis was performed using CYTOF toolkit (Fluidigm, San Francisco, CA, USA), and acquired simultaneously as a single sample.

**Real-time RT-PCR**

Total cellular RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Real-time RT-PCR was performed on an ABI7500 Thermocycler using Platinum ThermoScript One-Step system (Invitrogen, Zug, Switzerland). All mouse gene-specific mRNA levels were determined using commercial TaqMan GeneAssays (Applied Biosystems, Zug, Switzerland): Eif1a (Mm00456651_m1), Sucnr1 (Mm00519024_m1), Tnf (Mm00443258_m1), Il1b (Mm00434228_m1), Il4 (Mm00445259_m1), Il5 (Mm001290072_g1), Il13 (Mm00434206_g1), Il17 (Mm00521423_m1) and Il6 (Mm99999701_m1). For measurement of human EF1A mRNA, primers and probes (Eurogentec, Liege, Belgium) were designed with PRIMER EXPRESS software (Applied Biosystems, Zug, Switzerland) (forward, 5'-TTTGGAGACCGCAGCACTAGTGACCT-3'; reverse 5'-TCAGCCTGAGATGTCCCTGTAA-3'; probe 5'-TCAT TGATGCCCCAGGACACAGAC-3'). The expression of gene-specific human SUCNR1 mRNA was measured with commercial TaqMan GeneAssays kits (Applied Biosystems, Hs00263701_m1).

**Mass cytometry by time of flight**

Mass cytometry by time of flight (CyTOF) analysis on spleens, inguinal lymph nodes, blood and bone marrow of wt and Sucnr1<sup>−/−</sup> mice was performed as previously described (23) using a cocktail of monoclonal antibodies, conjugated to monoisotopic lanthanides (Table S1). All samples (three wt and three Sucnr1<sup>−/−</sup>) isolated from a given tissue were bar-coded using the Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm, San Francisco, CA, USA), and acquired simultaneously as a mixture to correct for fluctuations in signal strength during acquisition time. Data analysis was performed using CYTOBANK (Mountain View, CA, USA), SPOTFIRE (Palo Alto, CA, USA) software and R-based algorithms.

**Human cell isolation and differentiation**

Peripheral blood mononuclear cells were isolated using standard Ficoll density gradients from peripheral blood of healthy volunteers obtained from the Swiss Red Cross after signing informed consent. Human mast cells were...
differentiated from hematopoiestic precursors (Lonza, Switzerland) as described by Rädinger et al. (24).

**Generation and activation of mouse bone marrow-derived mast cells**

Bone marrow (BM) cells were obtained by flushing the humeri, femurs and tibias of wt and *Sucnr1*+/− mice with HBSS (Gibco, Zug, Switzerland) and subsequently cultured for 4–8 weeks using RPMI-1640/glutaMAX medium supplemented with 10% FCS, 1% penicillin/streptomycin, 1% MEM essential amino acids, 50 μM β-ME, 1 mM sodium pyruvate (all from Gibco) and 20 ng/ml IL-3 (Novartis, Basel, Switzerland). To investigate the role of GPR91 on mast cell development, BM cells were cultured in X-Vivo 15 medium (Gibco) supplemented with 1% MEM essential amino acids, 50 μM β-ME, 1 mM sodium pyruvate and 20 ng/ml IL-3 in the presence or absence of 50 μM succinate. After 4 weeks of cultivation, BM-derived mast cells (BMMC) were analyzed by flow cytometry using CD11b and CD117 antibodies (BD Biosciences). The purity of the BMMC was always >98%. BMMC were generally stimulated for 1–4 h in 96-well flat-bottom plates at a density of 2 × 10^5 cells/well using supplemented RPMI-1640/glutaMAX medium and 2 μg/ml IgE (BD Biosciences) and 0.1 μg/ml DNP-BSA as antigen (Sigma) in the absence or presence of succinate. At indicated time points, cells were harvested and processed for mRNA expression using cytokine-specific primers and probes (as described above). To monitor mast cell degranulation by the measurement of the release of the granule component, β-hexosaminidase, BMMC were washed with RPMI-1640/glutaMAX (without phenol red) supplemented with 25 mM HEPES, 0.1% BSA, 1% penicillin/streptomycin, 1x sodium pyruvate, 1x MEM essential amino acids and 50 μM β-mercaptoethanol and cross-linked for 1 h with 2 μg/ml IgE and 0.1 μg/ml DNP-BSA as the antigen. Cells were centrifuged, and supernatants were incubated for 30 min with a substrate 4-methylumbelliferyl-N-acetyl-β-glucosaminide (Sigma-Aldrich) diluted in citrate buffer and measured using white OptiPlate (PerkinElmer, Schwarzenbach, Switzerland).

Some cases, BMMC were transfected using specific siRNA (Aldrich) diluted in citrate buffer and measured using white

Statistics

One-tailed Mann–Whitney *U*-test or two-tailed Student’s *t*-test was used to analyze results. Significant differences with *P* < 0.05, *P* < 0.01 and *P* < 0.001 are illustrated as *, ** and ***, respectively.

**Results and discussion**

The skin is an effective barrier used by all vertebrates as an interface with the environment to provide the first line of defense from external factors. Infectious agents, toxins or trauma can harm the skin, causing the necrotic secretion of internal cell metabolites, which contribute to the activation of DC and the initiation of immune responses (25). In this context, we aimed to test the hypothesis that GPR91 expressed on epidermal Langerhans and dermal DC potentiates the development of ACD by sensing succinate release following tissue damage in the skin. We compared the reactions of wt and *Sucnr1*+/− mice to a classical ACD challenge (i.e. using oxazolone as the inducing hapten and measuring ear thickness as the primary readout). We hypothesized that hypersensitive responses to oxazolone would be lowest in *Sucnr1*+/− mice. Surprisingly, the extent of the responses observed in *Sucnr1*+/− mice following oxazolone challenge was consistently higher than that displayed by the wt counterparts (Fig. 1A,B). This result was confounding, as we had postulated earlier that antagonizing GPR91 would be beneficial for the treatment of several immune disorders (16) and such a reaction could compromise the safety of putative GPR91-based therapeutics. On the other hand, skin hyperreactivity was not augmented during the sensitization procedure with oxazolone and was reduced, rather than enhanced, following an ICD challenge (Fig. S2) of naïve *Sucnr1*+/− mice with TPA. Furthermore, the strong hypersensitivity of *Sucnr1*+/− mice to oxazolone did not correlate with an underlying increased T-cell response, either measured as T-cell infiltration (not shown) or measured as cutaneous transcript expression for classical Th2 cytokines like IL-4 and IL-13, which were rather reduced in the challenged gene-deficient mice (Fig. 1C). Of note, mRNA expression for the innate inflammatory cytokines IL-1β and TNF-α was not elevated in the ears of challenged *Sucnr1*+/− mouse group (Fig. S3A), which also did not display signs for exacerbated Th1, Th2 or Th17 cell differentiation, as assessed by the transcript expression of signature cytokines in the ear draining lymph nodes (Fig. S3B).

A closer look at the hematoxylin staining presented in Fig. 1A revealed a severe intradermal and subcutaneous edema in the ears of the hapten-challenged *Sucnr1*+/− mice, which is indicative for mast cell degranulation. RT-PCR analysis of both mouse and human mast cells differentiated in vitro from corresponding bone marrow precursors revealed copious transcripts encoding GPR91 (Figs 1D and S4). These observations prompted us to compare the in vitro activation of mast cells differentiated from the bone marrow of wt and *Sucnr1*+/− mice. As shown in Fig. 1E, mast cells from both, wt and *Sucnr1*+/− mice, express TNF-α mRNA, following in vitro activation with IgE cross-linked by a specific antigen. TNF-α expression was maximal at 1 h after activation and decreased progressively in the subsequent 4 h. However, at all time points examined, the magnitude of the response displayed by the mast cells derived from the *Sucnr1*+/− mice was higher than that observed from wt counterparts. To further assess the in vivo biological relevance of the enhanced activation displayed by *Sucnr1*+/− mast cells, we monitored blood vessel permeability following a PCA test. As demonstrated in Figs 1F and S5, *Sucnr1*+/− mice displayed exacerbated blood vessel leakage, as shown by the release of the
Evans blue following cross-link of i.d. injected DNP-specific IgE by the specific hapten. The responses were specific, as they were not elicited at the dermal sites injected with PBS (Fig. S5). On the other hand, the PCA test is an artificial setting that simply recapitulates the mechanistic mast cell activation induced in vitro, and thus, the potential risk for allergic hypersensitization associated with Sucnr1 deficiency was further assessed using an OVA-specific sensitization and challenge procedure, which mimics the allergic response associated with asthma. The macrophages present in the BAL of OVA-aerosol-challenged mice were less abundant in the Sucnr1/−/− mouse group (Fig. 2A). Lymphocytes, neutrophils and eosinophils, as well as overall cell counts, in the BAL of OVA-aerosol-challenged Sucnr1/−/− mice were always reduced with respect to BAL from wt mice (Fig. 2A,B), even though the results did not reach statistical significance. By contrast, the BAL levels of IgE (Fig. 2C), but not IgG (not shown), were significantly reduced in the Sucnr1/−/− group. Concurrently, serum IgE but not IgG was also reduced in Sucnr1/−/− mice (not shown). These results are in agreement with our previous report (16) implicating succinate as an endogenous adjuvant for T-cell-mediated responses.

The above data indicate that the absence of GPR91 signaling during the sensitization phase of immediate type I hypersensitivity responses reduces the development of T-cell-dependent IgE production and thus minimizes the risk of anaphylaxis upon subsequent encounter with the allergen. However, these experiments will not predict the risk of exacerbated mast cell activation mediated by GPR91 antagonists in an environment where preexisting immunocomplexes can...
drive pathology. Furthermore, bronchial hyperreactivity is not necessarily driven by mast cells in the C57BL/6 background, particularly if the challenge procedure is not repeated over a lengthy period of time (26). Thus, elucidating the threat of anaphylaxis would require specific and bioavailable low molecular weight antagonists of GPR91, which, to the best of our knowledge, are not yet available. We envisioned an alternative experimental condition in the mouse arthritis model induced by the transfer of the autoantibodies, directed to glucose-6-phosphatase isomerase, that develop in K/BxN mice (27). Passive transfer of serum from K/BxN mice to healthy wt recipient induces arthritis without the need to involve the generation of an adaptive response (27). The mechanism of this pathogenic antibody-induced disease has been investigated in detail, revealing major roles for IL-1β, TNF-α (28), FcγRIII/CD16 (29, 30) and C5aR/CD88 (29). In addition, the observation that mice with functional mast cell deficiency are protected from K/BxN serum induced arthritis and that disease susceptibility is restored in these mice following reconstitution with normal mast cells has been used as a strong argument implicating mast cells in this pathologic process (30, 31). Thus, we anticipated that if the lack of Sucnr1 predisposes for exacerbated mast cell function, the arthritic disease induced by immunization with methylated bovine serum albumin (mBSA), which is mechanistically an antigen-specific and T-cell-dependent (32) pathology, should be reduced in Sucnr1−/− mice, in accordance with our previous observations (16). Unexpectedly, the swelling of the paws from Sucnr1−/− mice transferred with K/BxN serum was significantly reduced in comparison with the paw swelling in wt mice (Fig. 3A). Disease amelioration in Sucnr1−/− mice correlated with prevention of joint inflammation and with reduction in IL-1β mRNA expression in popliteal lymph nodes draining the inflamed knees (not shown). Conversely, the results obtained in the AIA model were as predicted. Sucnr1−/− mice mounted diminished adaptive responses, and hence, they were less susceptible than wt animals to develop arthritis following challenge with mBSA (Fig. 3B). Together, these results indicate that the potential exacerbation of mast cell activation, which results from deletion of Sucnr1 gene, does not worsen mast cell-dependent arthritis. This apparent paradox could be explained by the observation that mast cells are crucial for the initiation of arthritis in the K/BxN serum transfer model but they are dispensable for disease progression, which requires a subsequent recruitment and effector function of macrophages (33).

Classic pathway activation of macrophages is associated with a metabolic switch from oxidative phosphorylation to glycolysis (34), similar to the changes occurring in tumors (35). Succinate dehydrogenase (SDH) catalyzes the conversion of succinate and flavin adenine dinucleotide quinone to fumarate. Absence of GPR91 signaling ameliorates arthritis Rubiö-Schneider et al.
(FAD) into fumarate plus FAD hydroquinone (FADH₂), which is coupled to the reduction of ubiquinone in the mitochondrial electron transport chain. Reduced oxygen or increase nutrients, as observed during hypoxia or in obesity, limits the activity of SDH resulting in the accumulation of succinate (36), which is ultimately exported to the cytosol and subsequently to the extracellular environment. Succinate inhibits hypoxia-inducible factor-1α (HIF-1α) prolyl hydroxylation and therefore stabilizes HIF-1α (37), which in turn enhances IL-1β production. Recently, Tannahill et al. (38) have shown that IL-1β production by activated macrophages is linked to the accumulation of succinate in the cytoplasm of these cells and we have observed that this mechanism is reinforced by the autocrine and paracrine reuptake of secreted succinate by GPR91 expressed on macrophages (39). Thus, although Sucnr1−/− mast cells could potentially augment initiation of arthritis, these effects are counteracted by the reduced IL-1β production by Sucnr1−/− macrophages.

The above findings are in line with our previous report, describing succinate as an alarmin (16). The only apparent contradiction was the observed hyperreactivity of Sucnr1−/− mast cells, which, although it did not translate into augmented disease, must have a physiological significance. Currently, we can only speculate that mast cells use GPR91 as a homeostatic receptor during their development. Indeed, mast cells from Sucnr1−/− mice displayed hypomorphic aspects when compared to equivalent cells from wt mice (Fig. 4A). To search for evidence of the presumed developmental defect, we differentiated mast cells from bone marrow precursors of wt mice in the absence of succinate using serum-free medium. Such differentiation process was very challenging in comparison with standard protocols performed in FCS-containing medium. Nevertheless, mast cells differentiated under these conditions and became stimulated in the presence of allergen and specific IgE. We found that the amount of TNF-α or β-hexosaminidase secreted by activated mast cells differentiated in the absence of succinate was higher than that produced by mast cells derived from the same precursors differentiated in the presence of 50 μM succinate (Fig. 4B). Thus, the absence of succinate during in vitro mast cell development recapitulated Sucnr1 deficiency. On the other hand, we could not exacerbate cytokine release by wt mast cells differentiated in normal culture medium, which were subsequently devoid of GPR91 signaling by exposure to Sucnr1-specific siRNA (Fig. 4C), further suggesting that the abnormal mast cell response was linked to the development of these cells.
Here, we have confirmed the role of GPR91 augmenting immune responses, and simultaneously, we have discovered that mast cell development in the absence of GPR91 signaling could lead to a hyperreactive reaction. This effect was apparent in the ACD model but not appreciated in the OVA-aerosol challenge or in the arthritis models, particularly in the K/BxN model, which is driven by pathogenic autoantibodies. While these results do not eliminate completely the risk of adverse effects mediated by mast cells developing with little GPR91 agonism, they suggest that the maintenance and progression of antibody mediated diseases are not crucially dependent on mast cell function. Of note, the phenotype imposed by genetic deletion of Sucnr1 is expected to be more penetrant than that enforced by pharmacologic antagonism of GPR91, which could be transient and affecting predominantly DC- and macrophage-mediated enhancement of adaptive effector responses.

**Conclusion**

This study reports the expression of GPR91 on mouse and human mast cells and reveals a hyperactive behavior of mouse *Sucnr1−/−* mast cells in a mechanistic *in vivo* model of skin inflammation. While such an effect potentially poses a threat for the development of therapeutic antagonists aiming to interfere with adaptive immune responses, subsequent investigations using animal models of asthma and arthritis suggest that the abnormal reactions of *Sucnr1−/−* mast cells are manifestations linked to their development in the absence of succinate signaling and do not exacerbate autoimmune pathology. Overall, our data support the use of GPR91 antagonists for the treatment of autoimmune diseases and particularly for rheumatoid arthritis.
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Author contributions

All authors contributed to the execution and analysis of the experiments as well as to the writing of the manuscript and approved its final version. TR-S and JMC designed, analyzed and interpreted all experiments. NC-P, GL and SJ performed ACD, asthma and PCA in vivo experiments. CRe, LR, BC and RK performed in vitro mast cell differentiation and activation experiments. CRe and LR performed the CyTOF experiments. JD, GW and AL-E performed arthritis in vivo experiments.

Conflicts of interest

All authors listed in this manuscript are (or were) employees of NIBR and are engaged in drug development activities.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. CyTOF analysis of wt and Sucnr1<sup>−/−</sup> mice.

Figure S2. ICD challenge of wt and Sucnr1<sup>−/−</sup> mice.

Figure S3. mRNA expression in oxazolone-challenged ears and draining lymph nodes from wt and Sucnr1<sup>−/−</sup> mice.

Figure S4. SUCNR1 mRNA expression in human hematopoietic stem cell-derived mast cells.

Figure S5. Macroscopic PCA response in wt and Sucnr1<sup>−/−</sup> mice.

Table S1. List of metal-tagged mAbs used in CyTOF experiments.

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