

1 Nrf2 pathway modulates Substance P induced human mast cell activation and 2 degranulation in the hair follicle

3 **Capsule summary:** Activation of Nrf2 in human mast cells exposed to Substance P induced
4 oxidative stress suppresses pro-inflammatory gene transcription, activation and
5 degranulation.

6 **Key words:** mast cells, Nrf2, oxidative stress, hair follicle, Substance P

7 **Abbreviations:** Nrf2 (nuclear factor (erythroid-derived 2)-like-2); SFN (Sulforaphane); MC
8 (mast cell); CTS (connective tissue sheath); HO-1 (heme-oxygenase 1); NQO1 (NADP(H)
9 dehydrogenase, quinone 1); PGD₂ (Prostaglandin D2); PGE₂ (Prostaglandin E2); SP
10 (Substance-P); PBDMCs (Peripheral blood derived mast cells); **HF (hair follicle); ROS**
11 **(reactive oxygen species).**

12 **To the Editor:**

13 Mast cells (MCs), are immune cells distributed throughout various tissues that respond to
14 allergic and inflammatory reactions¹. The neuropeptide **Substance P (SP) is a stimulus that**
15 **generates damaging reactive oxygen species (ROS) via activation of neurokinin-1 (NRK-1)**
16 **and G-protein couple receptor MRGPRX2 and causes MC degranulation^{2,3}.** This SP-induced
17 MC degranulation can result in the excessive accumulation of ROS causing inhibition of hair
18 growth^{3(S1-S3)}.

19 Importantly, IgE-induced MCs degranulation in the RBL-2H3 cell line can be suppressed by
20 activation of the Nrf2 (nuclear factor (erythroid-derived 2)-like 2) target gene, heme-
21 oxygenase 1 (HO-1)⁴, therefore we were interested in the role of Nrf2 in the SP-mediated
22 activation of primary human MCs *in situ* and *in vitro*. Nrf2 controls cellular responses to
23 oxidative stress by transcriptional regulation of antioxidant genes^(S4). The connective tissue
24 sheath (CTS) of human HFs contains MCs, **which allows investigation of human MC**
25 **phenotype and activities under physiologically relevant conditions *ex vivo*^{5(S5)}.** We **examined**
26 **whether the Nrf2 transcriptional pathway can be activated within human MCs and whether**
27 **this modulates SP-induced MC degranulation and secretory activity *in vitro* and *in situ*.**

28 Nrf2/Mast Cell Tryptase (MCT) double immuno-staining was performed on human scalp HF
29 sections. Intramesenchymal Nrf2 protein expression in the CTS was low (Figure 1A) **and**
30 **treatment with SFN did not increase Nrf2/MCT double+ cell number (Figure 1B). Recently**
31 **we demonstrated Nrf2-mediated upregulation of HO-1 in human HF CTS by the Nrf2**
32 **activator, Sulforaphane (SFN)⁶.** Therefore, isolated human HFs were pre-treated with SFN
33 for 24 hrs followed by SP stimulation for 4 hrs to activate native CTS MCs *ex vivo*. This
34 significantly increased the number of HO-1/MCT double-positive MCs in the CTS (Figure
35 1C-D). **Importantly, stimulation with SP did not increase the number of MCs, as it has been**
36 **shown in previous report^(S1).**

37 Next, Nrf2 activation by SFN, in primary human peripheral blood-derived cultured MCs
38 (PBDMCs) **was investigated.** An increase in Nrf2 phosphorylation is observed following
39 activation and is associated with increased nuclear translocation and transcriptional

40 activity^(S4). Using immunohistochemistry, Nrf2 displayed both cytoplasmic and nuclear
41 localisations under basal conditions, whilst SFN treatment increasing nuclear Nrf2
42 accumulation (Figure 1E-F). However, in LAD2 Nrf2 localisation was only cytoplasmic in
43 vehicle and SFN treated conditions (Supplementary Figure 1).

44 We asked whether the expression of Nrf2 target genes and pro-inflammatory MC genes is
45 modulated by SFN 24 hrs or SP 6 hrs in PBDMCs. A significant increase in the mRNA
46 expression of the Nrf2 target genes HO-1 and NADP(H) dehydrogenase-quinone 1 (NQO1)
47 (Figure 1G-H) was observed following SFN treatment. SP induced significant mRNA
48 transcription of the pro-inflammatory cytokine IL-1 β (Figure 1I), followed by reduction prior
49 SFN treatment. However, we did not observe the same transcriptional effect of the MC
50 activation marker CD69^(S8) or TNF- α (Supplementary Figure 2A-B). In LAD2 similar effect
51 was detected (Supplementary Figure 3).

52 Furthermore, we investigated whether Nrf2 activation could modulate SP-induced MC
53 degranulation. In LAD2 cells, SP triggered high surface expression of MC degranulation
54 markers CD107a and CD63 by nearly 70%, while SFN alone or prior SP 1 had no effect
55 (Supplementary Figures 4A-B). We also measured well-established β -hexominidase release,
56 yet no effect was detected (Supplementary Figure 4C). Therefore, PBDMCs were
57 investigated next and while their surface expression of CD63 and CD107a after SP
58 stimulation was approximately 30%, pre-treatment of PBMCs with SFN prior SP treatment
59 significantly reduced SP-stimulated of both markers (Figure 2A-C). Furthermore, SP induced
60 β -hexominidase release, whilst pre-treatment with SFN reduced β -hexominidase levels
61 (Figure 2D). Pre-treatment with SFN for 24 hrs did not show this protective effect
62 (Supplementary Figure 5A-B). Importantly, the inhibitory effect of SFN varies in intensity
63 between PBDMCs cultures. Previously, Matsushima *et al.* (2009) determined that
64 upregulation of HO-1 by the Nrf2 activator quercetin occurs rapidly, resulting in suppression
65 of degranulation in RBL-2H3 cells. It may well be that the 1 hr stimulation with SFN used in
66 this study stimulates a similarly rapid increase in HO-1 expression and enzymatic activity to
67 suppress SP induced degranulation⁴.

68 MCs are major source of Prostaglandin (PGE₂) and Prostaglandin D2 (PGD₂) production⁷ and
69 the release can occur very quickly upon stimulation^(S9). We investigated Nrf2 activation by
70 SFN for 1 hr (Figure 2E-F) or 24 hrs (Supplementary Figure 6A-B) impact on the 1hr SP-
71 induced secretion for these PGs into the PBDMCs supernatant by ELISA. A significant
72 increase in PGD₂ release occurred following SP treatment, whereas pre-treatment with SFN
73 prior to SP reduced this, though this did not reach the level of significance (Figure 2E). PGE₂
74 release was largely unaffected by either SFN pre-treatment or SP exposure (Figure 2F).
75 However, we found that SFN and SP treatment does not disturb the levels of prostaglandin
76 D2 synthase (PTGDs) and prostaglandin E2 synthase (PTGEs) at mRNA levels
77 (Supplementary Figure 6C-D).

78 Finally, we asked whether the SP-induced degranulation of native human skin MCs can be
79 suppressed by SFN *ex vivo*, using toluidine blue histochemistry. Results showed significant
80 SP-stimulated MC degranulation within the CTS, whilst SFN pre-treatment reduced not only

81 SP-stimulated degranulation but also the SP-induced increase in the total number of toluidine
82 blue⁺ human skin MCs (Figure 2G-I).

83 Our results provide the first evidence that human MCs exhibit substantial Nrf2 activity,
84 which can effectively suppress MC activation and degranulation induced by the neuropeptide
85 SP. Importantly, PGD₂ levels are increased in balding scalp HFs, whilst PGE₂ levels are
86 decreased^(S10-S11), therefore the intrafollicular balance between PGD₂ and PGE₂ has to be
87 controlled for optimal hair growth^{8(S12)}. Increased MC numbers/degranulation have been
88 reported in androgenic alopecia (AGA)⁸, and in the most common inflammatory hair growth
89 disorder, alopecia areata (AA), density, degranulation and proliferation of perifollicular MCs
90 are also increased^{9 (S13-14)}. The therapeutic targeting of Nrf2 in perifollicular MCs may be of
91 particular interest in the future management of both AGA and AA.

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110 **Figure 1: Nrf2 activity within human MCs.** (A) Nrf2 and MCT immunofluorescent
111 staining in isolated human HFs. MCT⁺ cells (green) showed low expression of Nrf2

112 immunofluorescence (red) with infrequent Nrf2/MCT double positive immunofluorescence.
113 (B) The number of Nrf2/MCT double+ cells did not increase after 2 hr and 4hrs (the graph
114 presents combined 2hrs and 4hrs treatment of SFN) (C-D) Treatment with SFN and SP
115 significantly increased expression of MCT/HO-1 double+ cells. MCT (purple) detected in the
116 CTS and HO-1 (red) activity was found in the HF epithelium and vasculature of CTS. (n=3;
117 data are mean \pm SEM; One-Way ANOVA; Significance indicated by *P<0.05;**P<0.01;
118 ***P<0.001). (E-F) Expression of phosphorylated nuclear Nrf2 (red) in human primary MCs
119 is increased after treatment with SFN (n=3; \pm SD). (G) HO-1, (H) NQO1, (I) IL-1 β , (J) CD69
120 in PBDMCs were measured by qRT-PCR and data reported as fold changes in normalized
121 expression (n=3; data are mean \pm SEM; RM-One Way ANOVA; Significance indicated by
122 *P<0.05; **P<0.01; ***P<0.001).

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124 **Figure 2: Stimulation with SP induces MC degranulation and upregulates secretion of**
125 **mediators.** (A) Gating strategy identifying expression of CD107a and CD63 in PBDMCs.
126 (B-C) Decrease of CD63 and CD107a PBDMCs after pre-treatment with SFN (D) Pre-
127 treatment with SP suppressed SP induced β -hexominidase release (n=7; data are mean
128 \pm SEM; paired t-test; Significance indicated by *P<0.05) (E-F) Secretion of lipid-derivatives
129 after treatment with SFN and SP (n=4-5; data are mean \pm SEM; One-Way ANOVA;
130 Significance indicated by *P<0.05) (G-I) Toluidine blue staining indicated MCs
131 degranulation and number within CTS of human HFs (n=3; data are mean \pm SEM; One-Way
132 ANOVA; Significance indicated by *P<0.05;**P<0.01; ***P<0.001).

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177 **References**

- 178 1. Bulfone-Paus S, Nilsson G, Draber P, Blank U, Levi-Schaffer F. Positive and Negative Signals in
179 Mast Cell Activation. *Trends Immunol.* 2017;
- 180 2. Fujisawa D, Kashiwakura J-I, Kita H, Kikukawa Y, Fujitani Y, Sasaki-Sakamoto T, et al.
181 Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with
182 severe chronic urticaria. *J Allergy Clin Immunol.* 2014;134:622–633.e9.
- 183 3. Liu N, Wang L-H, Guo L-L, Wang G-Q, Zhou X-P, Jiang Y, et al. Chronic restraint stress inhibits
184 hair growth via substance P mediated by reactive oxygen species in mice. *PLoS One.*
185 2013;8:e61574.
- 186 4. Matsushima M, Takagi K, Ogawa M, Hirose E, Ota Y, Abe F, et al. Heme oxygenase-1 mediates
187 the anti-allergic actions of quercetin in rodent mast cells. *Inflamm Res Off J Eur Histamine*
188 *Res Soc.* 2009;58:705–15.
- 189 5. Sugawara K, Biro T, Tsuruta D, Toth BI, Kromminga A, Zakany N, et al. Endocannabinoids limit
190 excessive mast cell maturation and activation in human skin. *J Allergy Clin Immunol.*
191 2012;129:726–738.e8.
- 192 6. Haslam IS, Jadkauskaite L, Szabo IL, Staeger S, Hesebeck-Brinckmann J, Jenkins G, et al.
193 Oxidative damage control in a human (mini-) organ: Nrf2 activation protects against oxidative
194 stress-induced hair growth inhibition. *J Invest Dermatol.* 2017;137:295–304.
- 195 7. Gaudenzio N, Sibilano R, Marichal T, Starkl P, Reber LL, Cenac N, et al. Different activation
196 signals induce distinct mast cell degranulation strategies. *J Clin Invest.* 2016;126:3981–98.
- 197 8. Nieves A, Garza LA. Does prostaglandin D2 hold the cure to male pattern baldness? *Exp*
198 *Dermatol.* 2014;23:224–7.
- 199 9. Bertolini M, Zilio F, Rossi A, Kleditzsch P, Emelianov VE, Gilhar A, et al. Abnormal interactions
200 between perifollicular mast cells and CD8+ T-cells may contribute to the pathogenesis of
201 alopecia areata. *PLoS One.* 2014;9:e94260.

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212 **Methods**

213 **Human HF collection**

214 Occipital scalp hair follicles (HFs) from male hair transplant surgeries were delivered from
215 the Farjo Medical Centre (Manchester, UK). **Male patients that undergone hair transplant
216 surgery were suffering from AGA, but the occipital scalp HFs are androgen-insensitive and
217 not affected by AGA, therefore suitable for the experimental procedures.** The tissue was
218 collected after informed patient consent according to the 'Declaration of Helsinki Principles'
219 together with institutional approval ethics from the University of Manchester.

220 **Human HF organ culture**

221 Isolated full-length scalp HFs in anagen VI were cultured in William's E media (Gibco ®,
222 Leicestershire, UK) supplemented with 2 mM of L-Glutamine (Invitrogen, Paisley, UK),
223 10ng/mL hydrocortisone (Sigma-Aldrich ®, UK), 1 µg penicillin/streptomycin antibiotic
224 mixture (Gibco®, Leicestershire, UK), insulin-transferin-selenium (Life technologies,
225 Paisley, UK) at 37°C 5 % CO₂ incubator^(S15-S16). Nrf2 activation was performed using 20 µM
226 of SFN (Sigma-Aldrich ®, UK) for 2 hours, 4 hours and 24 hours and 2 µM of SP (Milipore,
227 Nottingham, UK) for 4 hours.

228 **Immunofluorescence microscopy and immunohistochemistry**

229 Double staining of Nrf2/MCT was performed using PerkinElmer TSA plus Kit (PerkinElmer,
230 UK). Cryosections (6 µm) were fixed in 4 % PFA followed by permeabilisation in 0.1 %
231 Triton X. The blocking was performed using 3 % H₂O₂ (Sigma-Aldrich ®, UK) followed by
232 washes with TNT buffer. Additional blocking was done using TNB and primary Nrf2
233 antibody (Abcam 31163, Cambridge, UK) was left overnight at 4°C before incubation with
234 HRP goat-anti-rabbit (Life Technologies, Paisley, UK). Additional blocking was done using
235 Bloxall (Vector, Peterborough, UK) before addition of secondary MCT antibody (Abcam
236 2378, Cambridge, UK).

237 PBDMCs were dried at room temperature after stimulation with SFN (5 µM) for 2 hrs and
238 stained for Nrf2 phospho-S40 (Abcam76026, Cambridge, UK). Washes were done using TBS
239 + 0.1 % Tween 20. The blocking was performed using normal goat serum (NGS) 10 % and
240 primary antibody was diluted 1:100 followed by overnight incubation. Subsequently,
241 secondary antibody AF594 (goat anti-rabbit) (Life Technologies, Paisley, UK) 1:200 was
242 added for one hour, followed by washes and counterstain with DAPI.

243 MCT/HO-1 double-immunostaining was performed using Vector Immpress kit (Vector,
244 Peterborough, UK). Slides were blocked using Bloxall followed by incubation with 2.5 %
245 normal horse serum and primary HO-1 antibody (Sigma HPA000635, Sigma-Aldrich ®,
246 UK). Washes were performed using PBS and secondary MCT antibody (Abcam 2378,

247 Cambridge, UK) was used. Toluidine blue histochemistry (Tol) was performed by fixing
248 slides in acetone, followed by washes in water before staining in Tol blue working solution
249 for 3 minutes. The analysis of immunostaining was performed using a Biozero-8000
250 microscope (Keyence, Milton Keynes, UK) and staining analysis was quantified by ImageJ
251 software (NIH). **Degranulated MCs were identified by quantifying 5 or more extracellular**
252 **metachromatic granules in the direct vicinity of a clearly identifiable perifollicular MCs^{5(S5)}.**

253 **Primary human peripheral blood-derived cultured MCs (PBDMCs)**

254 Human peripheral blood was obtained from a blood bank in Manchester. Mononuclear cells
255 were obtained and CD117+ progenitor cells were isolated by positive selection of FcRI
256 block/CD117+ (Miltenyi Biotec GmbH, Surrey, UK) by magnetic cell sorting^(S17). For the
257 first four weeks cells were cultured in StemSpam medium supplement with 1 %
258 penicillin/streptomycin (Invitrogen, Paisley, UK), 50 ng/ml IL-6 (Peprotech, UK), 10 ng/mL
259 IL-3 (Peprotech, UK), 100 ng/mL Stem Cell factor (Peprotech, UK) and 10 µg/mL of LDL
260 (StemCell Technologies, Cambridge, UK). After four weeks cells were cultured in IMDM
261 medium (ThermoFisher, Paisley, UK) supplemented with 50 µM 2b-mercaptoethanol (Sigma-
262 Aldrich®, UK), 0.5 % BSA (Life Technologies, Paisley, UK), 1 % insulin – transferrin –
263 selenium (Life Technologies, Paisley, UK), 1 % penicillin/streptomycin (Invitrogen, Paisley,
264 UK), 50 ng/mL IL-6 (Peprotech, UK) and 100 ng/mL stem cell factor (rhSCF) (Peprotech,
265 UK). Cell viability and maturity after eight weeks was measured using fluorochrome-
266 conjugated antibodies FcεRI (Biolegend, London, UK) and CD117 (Biolegend, London, UK)
267 by FlowCytometry.

268 **LAD2 cell line culture**

269 Laboratory of Allergic Diseases 2 (LAD2) cells (kindly supplied by the NIAID, USA: Arnold
270 S. Kirshenbaum, and Dean D. Metcalfe) were cultured in StemPro-34 serum-free medium
271 (Invitrogen, Paisley, UK) supplemented with 100 U/mL penicillin/streptomycin (Invitrogen,
272 Paisley, UK), 100 U/mL glutamine (Invitrogen, Paisley, UK) and 100 ng/mL rhSCF
273 (Peprotech, UK)^(S7).

274 **MC degranulation and activity assay**

275 MC activity and degranulation was measured using flow cytometry. Briefly, LAD2 cells and
276 PBDMCs (1.5×10^5) were seeded in 96-well plate and stimulated with 5 µM SFN (Sigma-
277 Aldrich®, UK) for 1 hr or 24 hrs followed by stimulation with 5µM SP (Milipore,
278 Nottingham, UK) for 1 hr. Subsequently, cells were resuspended in FACS-buffer (2%
279 newborn calf serum, 0.1% NaN₃, 2mM EDTA in PBS) and stained with fluorochrome-
280 conjugated antibodies CD63 and CD69 (Biolegend, London, UK) for 30 min at 4⁰C followed
281 by wash with Live/Dead® Fixable Blue dead cell stain kit for UV excitation
282 (LifeTechnologies, Paisley, UK). Samples were analysed with a LSR II or Fortessa (BD
283 Biosciences, Oxford, UK).

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288 **RNA isolation and RT-PCR**

289 LAD2 cells or PBDMCs (1.5×10^5) were treated with 5 μ M SFN for 24 hrs followed by
290 stimulation with 5 μ M SP for 6 hrs. Total RNA was extracted with an RNeasy Micro kit
291 (Qiagen, Manchester,UK) according to the manufacturer's instructions. Complimentary DNA
292 (cDNA) was reverse transcribed using Tetro[®]cDNA synthesis kit (Bioline,UK). Quantitative
293 – PCR was performed using StepOne[™] real time PCR system (Applied Biosystems,
294 Warrington,UK) using Taqman[®] fast advance master mix and probes (Applied Biosystems,
295 Warrington,UK). The following probes from AppliedBiosystems were HMOX1
296 (Hs01110250_m1), NQO1 (Hs02512143_s1), CD69 (Hs00934033_m1) **and most important**
297 **pro-inflammatory mediator in skin disorders IL-1 β (Hs00174097_m1). In addition, PTGDS**
298 **(Hs00168748_m1) and PTGES (Hs00610420_m1) were used.** Samples were run using the
299 StepOne Plus[™] Real-Time PCR system and associated software (Applied Biosystems,
300 Warrington UK), relative expression was quantified against the housekeeping gene PPIA
301 (Hs04194521_s1).

302 **MCs mediators release assay**

303 PBDMCs (1×10^5) were cultured with 5 μ M SFN for 1 hr and 24 hrs followed by stimulation
304 with 5 μ M SP for 1 hr. Secretion of PGD₂ and PGE₂ were measured according to the
305 manufacturer's instructions (CaymanChemical, Cambridge,UK).

306 50 μ L (25000 cells) from the LAD2 cell or PBDMC cultures were taken and centrifuged to
307 separate the supernatant and cell pellet. Cell pellets were lysed in 50 μ L media culture 1%
308 Triton X-100. β -hexosaminidase was measured in supernatant as well as in the cell pellet by
309 adding 100 μ L β -hexosaminidase substrate, 10 mM p-nitrophenyl N-acetyl-beta-D-
310 glucosamine (Sigma-Aldrich) in 0.1M Na₂HPO₄ buffer (pH4.5) for 2h at 37°C, 5% CO₂. The
311 reaction was stopped by adding 100 μ L of 0.2M glycine buffer (pH10). Optical density was
312 measured at 405 nm. hMC degranulation was assessed as % release of total β -hexosaminidase.

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