CONCISE REPORT

Inactivation of fatty acid amide hydrolase exacerbates experimental fibrosis by enhanced endocannabinoid-mediated activation of CB1

Katrin Palumbo-Zerr,[1] Angelika Horn,[1] Alfiya Distler,[1] Pawel Zerr,[1] Clara Dees,[1] Christian Beyer,[1] Enrico Selvi,[2] Benjamin F Cravatt,[3] Oliver Distler,[4] Georg Schett,[1] Jörg H W Distler[1]

► An additional supplementary Figure is published online only. To view this file please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2012-201823).

[1]Department of Internal Medicine III and Institute for Clinical Immunology, University of Erlangen-Nuremberg, Erlangen, Germany
[2]Department of Clinical Medicine and Immunological Sciences, University of Siena, Siena, Italy
[3]The Department of Chemical Physiology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California, USA
[4]Center of Experimental Rheumatology and Zurich Center of Integrative Human Physiology, University Hospital Zurich, Zurich, Switzerland

Correspondence to
Dr Jörg H W Distler, Department of Internal Medicine III and Institute for Clinical Immunology, University of Erlangen-Nuremberg, Ulmenweg 18, Erlangen D-91054, Germany; joerg.distler@uk-erlangen.de

KP-Z and AH contributed equally.

Accepted 20 July 2012
Published Online First 22 August 2012

ABSTRACT

Background Selective targeting of the cannabinoid receptors CB1 and CB2 by synthetic compounds has revealed opposing roles of both receptors in fibrosis.

Objectives To characterise the role of endogenous cannabinoids (endocannabinoids) and their predominant receptor in fibrosis.

Methods The levels of endocannabinoids in mice were modulated by pharmacological or genetic inactivation of the enzyme fatty acid amide hydrolase (FAAH). The predominant receptor for endocannabinoids was determined by selective inhibition of either CB1 or CB2. The extent of fibrosis upon challenge with bleomycin was determined by quantification of dermal thickness, hydroxyproline content and myofibroblast counts.

Results The expression of FAAH is decreased in systemic sclerosis fibroblasts. FAAH-deficient mice with strongly increased levels of endocannabinoids were more sensitive to bleomycin. Consistently, pharmacological inhibition of FAAH significantly exacerbated bleomycin-induced fibrosis. Inhibition of CB1 completely abrogated the profibrotic effects of FAAH inactivation. In contrast, inhibition of CB2 only modestly enhanced fibrosis, indicating that CB1 is the predominant receptor for endocannabinoids in experimental fibrosis.

Conclusions Increased levels of endocannabinoids induced by inactivation of FAAH worsen experimental fibrosis via activation of CB1. These findings highlight the profibrotic effects of endocannabinoids and suggest that CB1 maybe a more promising candidate for targeted treatments in fibrotic diseases than CB2.

INTRODUCTION

Cannabinoids are derivates of the Δ^9-tetrahydrocannabinol, the active psychotropic compound of marijuana. However, cannabinoids are synthesised by plants, but also within the human body.[1] Endogenous cannabinoids or endocannabinoids regulate neuronal activity, but also control activation, differentiation and proliferation of a variety of non-neuronal cell types.[1 2] Cannabinoid signalling is regulated by at least three different mechanisms: (i) the expression levels of the individual cannabinoids receptors CB1 and CB2,[3 4] (ii) the relative ratio of CB1 and CB2 in a certain cell type or tissue and (iii) the levels of endocannabinoids. The levels of endocannabinoids are predominantly regulated by their degradation. The rate-limiting enzyme for the degradation of endocannabinoids is the serine hydrolase fatty acid amide hydrolase (FAAH). The importance of FAAH for the turnover of endocannabinoids is highlighted by a more than 15-fold increased level of endocannabinoids in FAAH-deficient mice ($FAAH^{-/-}$).[5] The prominent role of FAAH in cannabinoid turnover fostered the development of highly selective small-molecule inhibitors of FAAH that have recently been evaluated for the treatment of pain in patients with osteoarthritis.

We and others have previously shown that CB1 and CB2 have distinct roles in fibrosis.[6–8] Inhibition of CB1 ameliorates bleomycin-induced fibrosis, whereas inactivation of CB2 enhances experimental fibrosis. Although these studies demonstrate that modulation of individual cannabinoid receptors affects experimental fibrosis, they do not elucidate the role of endocannabinoids. In contrast to many synthetic cannabinoids, endocannabinoids are not selective for CB1 or CB2. Owing to the opposing effects of CB1 and CB2, endocannabinoids thus may exert profibrotic or antifibrotic effects depending on their predominant receptor. Given that FAAH is the major regulatory enzyme that determines the levels of endocannabinoids, we used $FAAH^{-/-}$ mice and selective inhibition of CB1 and CB2 to study the role of the endocannabinoid system and identify the predominant receptor in experimental fibrosis.

MATERIAL AND METHODS

Bleomycin-induced dermal fibrosis in FAAH-deficient mice

$FAAH^{-/-}$ mice have been described previously.[5] $FAAH^{-/-}$ mice were backcrossed onto a C57Bl/6 background for at least six generations. Wild-type C57Bl/6 littermates ($FAAH^{+/+}$) were used as controls. Skin fibrosis was induced by bleomycin as described.[9]

Treatment with inhibitors of FAAH, CB1 and CB2

In subsets of experiments, mice were treated four times a day (qid) intraperitoneally (IP) with the FAAH inhibitor JNJ 1661010 (10 mg/kg), a combination of JNJ 1661010 (10 mg/kg/qid IP) and the CB1 antagonist AM281 (10 mg/kg/qid IP) or a combination of JNJ 1661010 (10 mg/kg/qid IP) and the CB2 antagonist AM630 (2.5 mg/kg/qid IP). JNJ 1661010, AM281 and AM630 were purchased from

Biozol (Eching, Germany). AM281 and AM630 have been proved to effectively inhibit CB1 and CB2, respectively, in mice in the doses used herein.[6 7 10] Treatment with those inhibitors started in parallel to bleomycin challenge.

Evaluation of dermal fibrosis

The extent of fibrotic changes was determined by (i) measuring the dermal thickness, (ii) quantification of myofibroblasts and (iii) analyses of the hydroxyproline content, as described.[9 11–14]

Immunofluorescence staining

Formalin-fixed, paraffin-embedded skin sections were stained for FAAH or CB1 (both Abcam, San Antonio, Texas, USA). Peroxidase labelled species-specific immunoglobulin antibodies (Dako, Glostrup, Denmark) were used as secondary antibodies. Images were captured at a 200-fold magnification.[15]

Statistics

All data are presented as median with IQR, and differences between the groups were tested for their statistical significance by non-parametric Mann–Whitney U test. A p value of <0.05 was considered statistically significant; p values are expressed as follows: $^{*}0.05>p>0.01$; $^{**}0.01>p>0.001$; $^{***}p<0.001$.

RESULTS

FAAH$^{-/-}$ mice are more sensitive to bleomycin-induced skin fibrosis

Bleomycin-induced dermal fibrosis was significantly more pronounced in FAAH$^{-/-}$ mice with highly increased levels of endocannabinoids (figure 1A). The mean increase in dermal thickening upon challenge with bleomycin in FAAH$^{-/-}$ mice exceeded dermal thickening in FAAH$^{+/+}$ mice by 79% ($p=0.004$) (figure 1B). The number of myofibroblasts and the hydroxyproline content were also significantly higher in FAAH$^{-/-}$ mice than in FAAH$^{+/+}$ mice injected with bleomycin (figure 1C,D).

Pharmacological inhibition of FAAH exacerbates bleomycin-induced fibrosis

To confirm the increased sensitivity of FAAH$^{-/-}$ mice to experimental fibrosis by a pharmacological approach, bleomycin-challenged wild-type mice were treated with the selective FAAH inhibitor JNJ 1661010. Treatment of wild-type mice with JNJ 1661010 resulted in prominent exacerbation of bleomycin-induced fibrosis with significantly increased dermal thickening, myofibroblast counts and hydroxyproline content compared with sham-treated, bleomycin-challenged mice (figure 2A–D).

CB1 antagonists reverse the profibrotic effects of FAAH inhibition

To identify the major receptor for the effects of endogenous cannabinoids, CB1 and CB2 were specifically blocked by AM281 and AM630 in addition to inhibition of FAAH by JNJ 1661010. Simultaneous inhibition of CB1 and FAAH completely abrogated the profibrotic effects of FAAH inhibition (figure 3A). The mean dermal thickness, myofibroblast counts and hydroxyproline content in mice treated with AM281 and JNJ 1661010 were significantly reduced compared with a single treatment with JNJ 1661010 (reductions by 45%, 47% and 50%, $p<0.003$ for all) and did not differ from those obtained for sham-treated, bleomycin-challenged mice (figure 3B–D). In contrast to the antifibrotic effects of the CB1 antagonist, inhibition of the CB2 receptor by AM630 further enhanced fibrosis. However, the relative effects were less pronounced than for CB1 inhibition with modest increases of dermal thickness, myofibroblast counts and hydroxyproline content compared with bleomycin-challenged mice treated with the FAAH inhibitor JNJ 1661010.

Decreased expression of FAAH in systemic sclerosis (SSc) fibroblasts

To confirm the relevance of our findings for the pathogenesis of SSc, we analysed the expression of FAAH and CB1 by immunohistochemistry. Staining for FAAH was decreased in

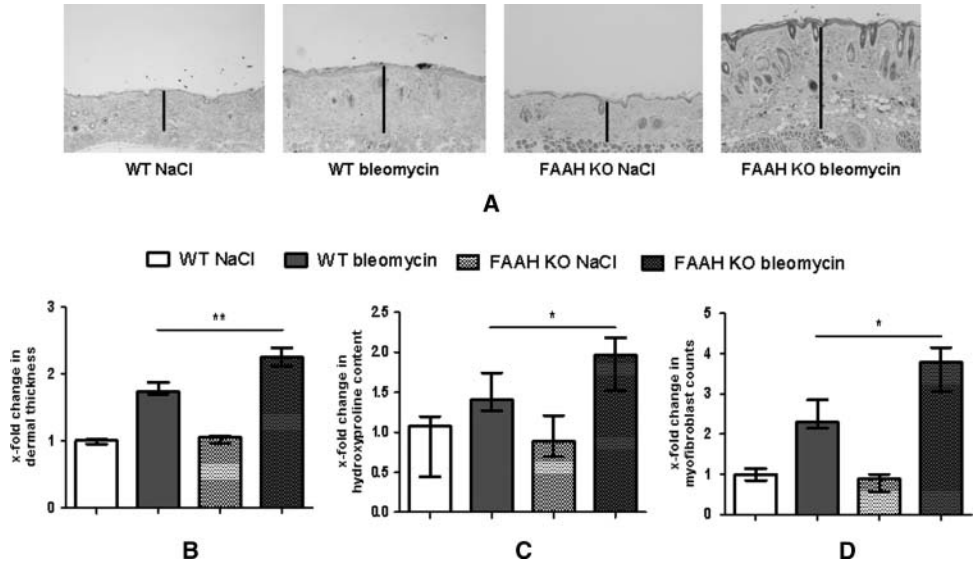

Figure 1 Fatty acid amide hydrolase (FAAH)$^{-/-}$ mice are more sensitive to bleomycin-induced dermal fibrosis. FAAH$^{+/+}$ mice injected with NaCl, FAAH$^{-/-}$ mice challenged with bleomycin, FAAH$^{-/-}$ mice injected with NaCl and FAAH$^{-/-}$ challenged with bleomycin were evaluated (n=5 per group). (A) Representative skin stained with haematoxylin/eosin at 100-fold magnification. (B) Dermal thickening. (C) Hydroxyproline content. (D) Myofibroblast counts. KO, knockout; WT, wild-type.

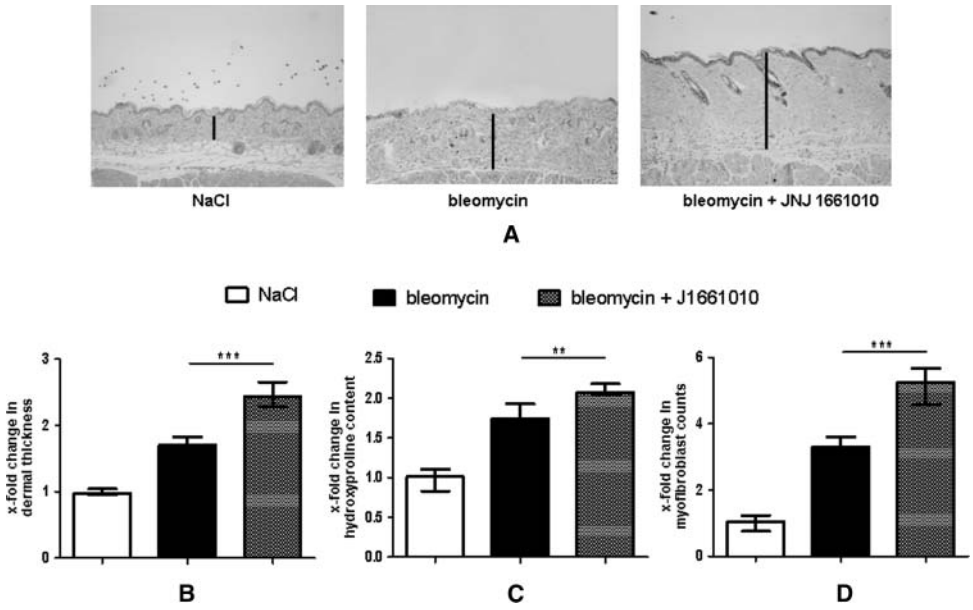

Figure 2 Treatment with the fatty acid amide hydrolase agonist JNJ 1661010 enhances experimental fibrosis. The outcome of mice treated intraperitoneally four times a day with 10 mg/kg JNJ 1661010 were compared with sham-treated, bleomycin-challenged mice and with sham-treated mice injected with NaCl (n=8 per group). (A) Representative skin stained with haematoxylin/eosin at 100-fold magnification. (B) Dermal thickening. (C) Hydroxyproline content. (D) Myofibroblast counts.

patients with SSc as compared with healthy individuals (see supplementary figure S1). Although double staining with markers of fibroblasts was not performed, the morphology of FAAH positive cells indicated that fibroblasts in healthy skin express FAAH. In contrast, FAAH was barely detectable in skin from patients with SSc. The differential expression of FAAH was confirmed in cultured fibroblasts with a 42±12% reduction of FAAH mRNA in SSc fibroblasts ($p<0.05$). CB1 staining was seen around inflammatory infiltrates in a subset of patients with SSc, whereas only few scattered cells stained positive for CB1 in the skin of healthy individuals.

DISCUSSION

The levels of endocannabinoids are mainly regulated at the level of degradation. FAAH is the rate-limiting enzyme for the degradation of endocannabinoids.[5] We demonstrate in this study that the increase in endocannabinoid levels exacerbates experimental fibrosis. Genetic or pharmacological inactivation of FAAH significantly increased dermal thickening, enhanced differentiation of resting fibroblasts into myofibroblasts and raised hydroxyproline levels upon challenge with bleomycin. Based on these findings, it can be speculated that an increase of the cannabinoid levels induced by cannabinoid abuse—for

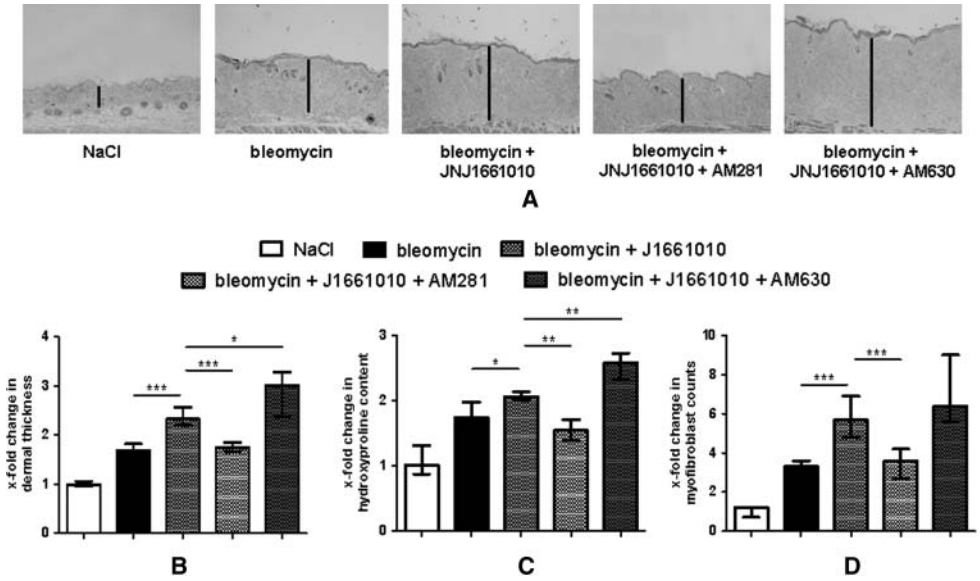

Figure 3 The effects of fatty acid amide hydrolase (FAAH) inactivation are predominantly mediated by CB1. The outcome of mice with simultaneous inhibition of either CB1 and FAAH (using AM281 and JNJ 1661010) or CB2 and FAAH (using AM630 and JNJ 1661010) was compared with bleomycin-challenged mice treated with JNJ 1661010 alone, sham-treated, bleomycin-challenged mice and sham-treated mice injected with NaCl (n=9–12 per group). (A) Representative skin stained with haematoxylin/eosin at 100-fold magnification. (B) Dermal thickening. (C) Hydroxyproline content. (D) Myofibroblast counts.

example, by smoking marijuana, may also enhance the sensitivity to fibrotic stimuli. Indeed, cannabinoid abuse was identified as a risk factor of progression of fibrosis in patients with chronic hepatitis C.[16] Although the expression of FAAH mRNA and protein is decreased in SSc, the levels of endocannabinoids upon pharmacological or genetic inactivation of FAAH probably exceed those in SSc and further studies in humans are needed to establish a general link between cannabinoid levels and fibrosis. These findings suggest that attention should be paid to cannabinoid abuse in patients with fibrotic diseases.

We and others demonstrated previously that CB1 and CB2 are both implicated in the pathogenesis of fibrotic diseases,[6–8 17–19] probably by regulating T cell function and Th1/Th2 responses.[20] To determine the predominant receptor that mediates the effects of increased endocannabinoids, bleomycin-challenged mice were simultaneous treated either with inhibitors of CB1 and FAAH or inhibitors of CB2 and FAAH. Inhibition of CB1 completely prevented the exacerbation of fibrosis upon FAAH inactivation. In contrast, inactivation of CB2 only modestly enhanced bleomycin-induced fibrosis. These findings demonstrate that CB1 is the dominant receptor for endocannabinoids in experimental fibrosis and that the enhanced activation of CB1 mediates the increased susceptibility to fibrosis upon increase of cannabinoid levels. For the development of targeted treatments, these findings may also indicate that inhibition of CB1 may be a more promising approach than activation of CB2, at least from a pathophysiological perspective. However, given the physiological roles of CB1—for example, in nociception and regulation of appetite, careful monitoring will be needed. Although evidence for a key role of cannabinoids in SSc is accumulating,[6–8 17–19] several key questions need to be answered: Are the levels of particular endocannabinoids altered in SSc? How are cannabinoids modulating the release of profibrotic mediators by leucocytes? Do endocannabinoids also play a role in vascular disease in SSc?

In summary, we demonstrate that enhanced endocannabinoid levels upon inactivation of FAAH worsen experimental fibrosis and that these effects are predominantly mediated by CB1. Our findings may have other implications as they suggest that (i) cannabinoid consumption should be avoided in fibrotic diseases and (ii) CB1 maybe a more promising candidate for targeted treatments in fibrotic diseases than CB2.

Acknowledgements We thank Madeleine Demleitner, Stefan Fritz, Maria Halter, Anna-Maria Herrmann and Verena Wäsch for excellent technical assistance.

Contributors AH, KP-Z, JHWD: design of the study; AH, KP-Z, AD, PZ, CD, CB: acquisition of data; AH, KP-Z, AD, PZ, CD, CB, CD, ES, OD, GS, JHWD: interpretation of data; AH, KP-Z, JHWD: manuscript preparation; BFC: provision of essential material.

Funding CB: research scholar at the Interdisciplinary Center of Clinical Research (IZKF) in Erlangen; grant from the Erlanger Leistungsbezogene Anschubfinanzierung und Nachwuchsförderung (ELAN). JHWD: grant A40 from IZKF in Erlangen; grants from the Deutsche Forschungsgesellschaft (grants DI 1537/2-1, DI 1537/4-1, DI 1537/5-1, AK 144/1-1 and SCHE 1583/7-1); and the Career Support Award of Medicine of the Ernst Jung Foundation.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Mackie K.** Cannabinoid receptors: where they are and what they do. *J Neuroendocrinol* 2008;**20**(Suppl 1):10–14.
2. **Bisogno T.** Endogenous cannabinoids: structure and metabolism. *J Neuroendocrinol* 2008;**20**(Suppl 1):1–9.
3. **Matsuda LA,** Lolait SJ, Brownstein MJ, *et al*. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 1990;**346**:561–4.
4. **Munro S,** Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 1993;**365**:61–5.
5. **Cravatt BF,** Demarest K, Patricelli MP, *et al*. Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA* 2001;**98**:9371–6.
6. **Akhmetshina A,** Dees C, Busch N, *et al*. The cannabinoid receptor CB2 exerts antifibrotic effects in experimental dermal fibrosis. *Arthritis Rheum* 2009;**60**:1129–36.
7. **Marquart S,** Zerr P, Akhmetshina A, *et al*. Inactivation of the cannabinoid receptor CB1 prevents leukocyte infiltration and experimental fibrosis. *Arthritis Rheum* 2010;**62**:3467–76.
8. **Teixeira-Clerc F,** Julien B, Grenard P, *et al*. CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nat Med* 2006;**12**:671–6.
9. **Distler JH,** Jungel A, Huber LC, *et al*. Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. *Arthritis Rheum* 2007;**56**:311–22.
10. **Mukhopadhyay P,** Batkai S, Rajesh M, *et al*. Pharmacological inhibition of CB1 cannabinoid receptor protects against doxorubicin-induced cardiotoxicity. *J Am Coll Cardiol* 2007;**50**:528–36.
11. **Akhmetshina A,** Dees C, Pileckyte M, *et al*. Dual inhibition of c-abl and PDGF receptor signaling by dasatinib and nilotinib for the treatment of dermal fibrosis. *FASEB J* 2008;**22**:2214–22.
12. **Beyer C,** Reich N, Schindler SC, *et al*. Stimulation of soluble guanylate cyclase reduces experimental dermal fibrosis. *Ann Rheum Dis* 2012;**71**:1019–26.
13. **Dees C,** Akhmetshina A, Zerr P, *et al*. Platelet-derived serotonin links vascular disease and tissue fibrosis. *J Exp Med* 2011;**208**:961–72.
14. **Bergmann C,** Akhmetshina A, Dees C, *et al*. Inhibition of glycogen synthase kinase 3beta induces dermal fibrosis by activation of the canonical Wnt pathway. *Ann Rheum Dis* 2011;**70**:2191–8.
15. **Palumbo K,** Zerr P, Tomcik M, *et al*. The transcription factor JunD mediates transforming growth factor {beta}-induced fibroblast activation and fibrosis in systemic sclerosis. *Ann Rheum Dis* 2011;**70**:1320–6.
16. **Hezode C,** Roudot-Thoraval F, Nguyen S, *et al*. Daily cannabis smoking as a risk factor for progression of fibrosis in chronic hepatitis C. *Hepatology* 2005;**42**:63–71.
17. **Garcia-Gonzalez E,** Selvi E, Balistreri E, *et al*. Cannabinoids inhibit fibrogenesis in diffuse systemic sclerosis fibroblasts. *Rheumatology (Oxford)* 2009;**48**:1050–6.
18. **Gonzalez EG,** Selvi E, Balistreri E, *et al*. Synthetic cannabinoid ajulemic acid exerts potent antifibrotic effects in experimental models of systemic sclerosis. *Ann Rheum Dis* 2012;**71**1545–51.
19. **Selvi E,** Lorenzini S, Garcia-Gonzalez E, *et al*. Inhibitory effect of synthetic cannabinoids on cytokine production in rheumatoid fibroblast-like synoviocytes. *Clin Exp Rheumatol* 2008;**26**:574–81.
20. **Klein TW,** Newton C, Larsen K, *et al*. Cannabinoid receptors and T helper cells. *J Neuroimmunol* 2004;**147**:91–4.

Inactivation of fatty acid amide hydrolase exacerbates experimental fibrosis by enhanced endocannabinoid-mediated activation of CB1

Katrin Palumbo-Zerr, Angelika Horn, Alfiya Distler, Pawel Zerr, Clara Dees, Christian Beyer, Enrico Selvi, Benjamin F Cravatt, Oliver Distler, Georg Schett and Jörg H W Distler

Ann Rheum Dis 2012 71: 2051-2054 originally published online August 22, 2012
doi: 10.1136/annrheumdis-2012-201823