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粘膜型マスト細胞特異的抗体ライブラリを用いたアレルギー・炎症性疾患の早期診断・治療法の開発

平成２４年度　総括研究報告書

研究代表者　國澤　純

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I. 総括研究報告書

厚生労働科学研究費補助金（医療機器開発推進研究事業）

総括研究報告書

粘膜型マスト細胞特異的抗体ライブラリを用いた免疫疾患の早期診断・治療法の開発

研究代表者：國澤　純（東京大学医科学研究所）

研究要旨：

近年患者数が爆発的に増加しているアレルギー炎症性疾患において、マスト細胞が主要エフェクター細胞の一つとして機能している。また最近の研究から、マスト細胞は画一な細胞ではなく、様々なサブセットから構成され、各々が異なる免疫反応に関わっていることが示唆されている。その中でアレルギー炎症性疾患の発症には粘膜型マスト細胞が強く関わっていることが提唱されていたが、マスト細胞サブセットという観点からのアレルギー炎症性疾患に対する診断・予防・治療法は開発されていないのが現状である。本研究課題においては、研究代表者がこれまで行ってきた粘膜組織におけるアレルギー炎症性疾患に関する知的・技術基盤と世界に先駆け独自に樹立した粘膜型マスト細胞特異的抗体ライブラリを駆使し、アレルギー炎症性疾患における早期診断技術と治療法の開発を進める。本事業の初年度であるH23年度は、樹立抗体の一つであるclone 1F11が炎症性腸疾患に対して有効であることが示すことに成功し、また本抗体の認識分子が細胞外核酸(ATP)受容体の一つであるP2X7受容体であることを見いだした。

本事業の最終年度にあたる24年度の研究においては、clone 1F11をプローブとして用い、炎症性腸疾患におけるマスト細胞およびP2X7の役割について検証した。はじめに、in vitroにおいて野生型マスト細胞をclone 1F11で前処理した後、ATPで刺激したところ、通常観察されるマスト細胞の活性化は認められなかった。同様に、P2X7欠損マスト細胞においても細胞外ATPに対する不応答が認められた。更にマスト細胞を欠損するマウスに野生型、もしくはP2X7欠損マスト細胞を再構築し、炎症性腸疾患モデルを適用したところ、野生型マスト細胞で再構築した群では腸炎の増悪化が観察されたのに対し、P2X7欠損マスト細胞で再構築した群においては、腸炎の発症が有意に抑制されていた。

これらの知見をもとに、ヒト炎症性腸疾患との関連についてヒト検体を用いた解析を行ったところ、炎症性腸疾患の一つであるクローン病の大腸組織において、マスト細胞上に発現するP2X7受容体が増強していることが明らかとなった。つまり、本抗体認識分子であるP2X7が、クローン病の病態形成を示唆するマーカーとなり得ることが新たに見出された。以上の結果は、炎症性腸疾患における早期診断・予防・治療技術としての粘膜型マスト細胞特異的抗体の有効性と新たな治療標的としてのP2X7の可能性を示すものであると考える。

本研究課題においては分担研究者を配していない。

A. 研究目的

近年患者数が爆発的に増加しているアレルギー炎症性疾患においてマスト細胞が主要エフェクター細胞の一つとして知られている。本研究では、アレルギー炎症性疾患の主要発症部位である粘膜組織に存在するマスト細胞を標的とした新規予防・診断・治療戦略の確立を目指した研究を遂行している。

一般にマスト細胞は結合組織型と粘膜型の２種類に大別され、粘膜型マスト細胞が粘膜組織におけるアレルギー・炎症性疾患に主に関わると考えられているが、その特異的分子や分化経路についてはほとんど解明されていない。このような背景のもと、これまでに研究代表者は粘膜型マスト細胞を特異的に識別できる抗体の樹立を試み、現在までに６種類のマスト細胞特異的抗体を樹立することに成功している。本事業においては研究代表者が世界に先駆け独自に樹立した粘膜型マスト細胞特異的抗体の認識分子の同定を進めていくと同時に、食物アレルギーや炎症性腸疾患モデルを用い、治療や予防、診断への応用を進める。これにより、粘膜型マスト細胞の特異性に立脚した早期診断技術と粘膜型マスト細胞を標的とした抗体療法の確立を目指す。

B. 研究方法

1. In vitro でのマスト細胞の活性化

in vitro で IL-3 と stem cell factor を用いて、野生型もしくは P2X7 欠損マウス由来の骨髄細胞よりマスト細胞(BMMC)を誘導した。BMMC を ATP(0.5mM)で刺激する際に clone 1F11 もしくはコントロール抗体で処理を行った。また、刺激後 20-30 分後に PBS を添加し反応を停止し、細胞表面の CD63 分子の発現率をフローサイトメトリー法で定量した。

2. マスト細胞再構築マウスにおける炎症性腸疾患に対する効果の検討

前述 1 の方法と同様に骨髄より誘導したマスト細胞(1x10^7 細胞)を、マスト細胞欠損マウスである $Kit^{W\text{-}sh/W\text{-}sh}$ マウスに尾静脈および腹腔に投与した。3 ヶ月後にトリニトロベンゼンスルホン酸（TNBS）の直腸投与による炎症性腸疾患モデルを適用し、マウスの体重変化を測定した。さらに TNBS 投与４日後に大腸組織から単核球を回収し、細胞表面での CD63 分子の発現を指標に c-kit と FcεRI 両陽性のマスト細胞の活性化をフローサイトメトリーにて測定した。

3. ヒト検体を用いた解析

大阪大学消化器内科との共同研究を行い、健常人、クローン病、潰瘍性大腸炎の腸管組織切片を入手した。抗ヒト P2X7 抗体と抗マスト細胞抗体を用いて組織染色を行った。

（倫理面への配慮）

動物実験、ならびにヒト組織解析は東京大学医科学研究所のガイドラインに則り、承認を得たプロトコールにて行った（承認番号２０－２８、２０－６７－０３３１）。

C. 研究結果

本事業における昨年度の研究から、clone 1F11 は炎症性腸疾患モデルにおいて体重減少など炎症性腸疾患に伴う病態を抑制できることを見いだした。さらにプロテオミクス解析から、clone 1F11 の認識抗原として細胞外核酸(ATP)の受容体の一つである P2X7 を同定した。これらの知見をもとに本年度は clone 1F11 の作用機序の解明、ならびに clone 1F11 の認識分子である P2X7 の役割について解析を進めた。骨髄由来マスト細胞を用いた in vitro の解析から、clone 1F11 で前処理したマスト細胞では、細胞外 ATP の刺激による活性化が抑制されることが示され（図 1)、昨年度 in vivo のモデルで観察されたマスト細胞の活性化抑制がマスト細胞に対する直接的作用であることが示された。更に P2X7 欠損マウスから骨髄由来マスト細胞を作製し、ATP 刺激による活性化を検証したところ、clone 1F11 前処理の結果と同様、P2X7 を欠損するマスト細胞では細胞外 ATP による活性化が起こらないことを示した。このことから、細胞外 ATP は P2X7 依存的にマスト細胞を活性化させることが明らかとなった。

また *in vivo* におけるマスト細胞と P2X7 の関与を検討する目的で、マスト細胞欠損マウスに野生型もしくは P2X7 欠損マスト細胞を再構築した。これらのマウスに TNBS 誘導性の炎症性腸疾患モデルを適用したところ、野生型マスト細胞で再構築した群では腸炎の増悪化が観察されたのに対し、P2X7 欠損マスト細胞で再構築した群においては腸炎の発症が有意に抑制されていた（図 2)。さらに、P2X7 欠損マスト細胞再構築群において、マスト細胞の活性化率が低下していることも確認した。

これらの知見をもとにヒト検体での解析を行った。代表的炎症性腸疾患であるクローン病と潰瘍性大腸炎の患者の方の大腸組織でのマスト細胞上の P2X7 の発現を確認したところ、クローン病患者の大腸においてマスト細胞の P2X7 の発現が増強していた（図 3)。一方で潰瘍性大腸炎の患者の大腸組織では P2X7 陽性マスト細胞はほとんど認められなかった。

D. 考察

本事業の２年目にあたる H24 年度は、初年度に炎症性腸疾患に対して有効であることが示された clone 1F11 から得られた知見を基盤に、clone 1F11 の認識分子である細胞外核酸(ATP)の受容体 P2X7 を介したマスト細胞の活性化と炎症性腸疾患との関連を明らかにした。マスト細胞の in vitro 培養系を用いた解析から、ATP による直接的作用による炎症性サイトカインや脂質メディエーターの産生が、マスト細胞による腸炎増悪化に関与している可能性が示された。またマスト細胞欠損マウスに P2X7 欠損マスト細胞を再構築したマウスを用いた解析において、野生型マウスを clone 1F11 で処理した際と同様に炎症性腸疾患の発症抑制が認められたことから、ATP/P2X7 を介したマスト細胞の活性化が炎症性腸疾患の発症において重要であることが示された。特に

P2X7 はマスト細胞以外にも樹状細胞やT細胞にも発現していることから、初年度の解析段階においてはマスト細胞以外の細胞を介して P2X7 依存的な炎症反応が関わっている可能性があったが、今回の結果からマスト細胞を介した ATP-P2X7 依存的な活性化が炎症性腸疾患の発症における重要な経路であることが示された。

さらには炎症性腸疾患の患者サンプルを用いた検討によって、クローン病患者の大腸組織において、マスト細胞上の P2X7 の発現が観察された。このことはヒトの炎症性腸疾患の発症においても P2X7 が関与していること、またマスト細胞上の P2X7 の発現を指標にした診断が可能であることを示唆する結果であると考える。一方、もう一つの代表的炎症性腸疾患である潰瘍性大腸炎においては、健常人と同様、マスト細胞上に P2X7 の発現が認められなかった。マウスのマスト細胞においては、恒常的な P2X7 の発現が見られるのに対し、ヒトにおいては P2X7 を発現しないのがデフォルトとなっており、クローン病の発症に伴い発現が増強することが明らかとなった。今後、マウスとヒトにおける発現機序の違いを認識しながら、発現制御メカニズムを解明することで、炎症性腸疾患に対する新たな治療戦略が提唱できるものと期待される。

E．結論

これまでに樹立したマスト細胞特異的抗体ライブラリを用いた検証から、マスト細胞に発現する P2X7 が炎症性腸疾患の発症に深く関わっている可能性を新たに見出している。またヒトクローン病においても、マスト細胞での P2X7 の発現が認められたことから、マスト細胞上の P2X7 の発現がクローン病の発症の指標診断として有効である可能性が示された。

F. 健康危機情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

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該当事項なし

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特記事項なし

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors

Yosuke Kurashima[1,2,3], Takeaki Amiya[1,3,4], Tomonori Nochi[1], Kumiko Fujisawa[1,3], Takeshi Haraguchi[5], Hideo Iba[5], Hiroko Tsutsui[6], Shintaro Sato[1,3], Sachiko Nakajima[7], Hideki Iijima[7], Masato Kubo[8,9], Jun Kunisawa[1,4] & Hiroshi Kiyono[1,2,3,4]

Mast cells are known effector cells in allergic and inflammatory diseases, but their precise roles in intestinal inflammation remain unknown. Here we show that activation of mast cells in intestinal inflammation is mediated by ATP-reactive P2X7 purinoceptors. We find an increase in the numbers of mast cells expressing P2X7 purinoceptors in the colons of mice with colitis and of patients with Crohn's disease. Treatment of mice with a P2X7 purinoceptor-specific antibody inhibits mast cell activation and subsequent intestinal inflammation. Similarly, intestinal inflammation is ameliorated in mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice, and reconstitution with wild-type, but not $P2x7^{-/-}$ mast cells results in susceptibility to inflammation. ATP-P2X7 purinoceptor-mediated activation of mast cells not only induces inflammatory cytokines, but also chemokines and leukotrienes, to recruit neutrophils and subsequently exacerbate intestinal inflammation. These findings reveal the role of P2X7 purinoceptor-mediated mast cell activation in both the initiation and exacerbation of intestinal inflammation.

[1] Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 108-8639, Japan. [2] Graduate School of Medicine, The University of Tokyo, 113-0033, Japan. [3] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Tokyo 102-0075, Japan. [4] Department of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo, Chiba 277-8561, Japan. [5] Division of Host-Parasite Interaction, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 108-8639, Japan. [6] Department of Microbiology, Hyogo College of Medicine, Nishinomiya 663-8501, Japan. [7] Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 565-0871, Japan. [8] Research Center for Allergy and Immunology, RIKEN, Yokohama Institute, Tsurumi, Yokohama, Kanagawa, 230-0045, Japan. [9] Division of Molecular Pathology, Research Institute for Biological Sciences, Tokyo University of Sciences, Chiba 278-0022, Japan. Correspondence and requests for materials should be addressed to J.K. (email: kunisawa@ims.u-tokyo.ac.jp) or to H.K. (email: kiyono@ims.u-tokyo.ac.jp).

Both active and quiescent immunity occur simultaneously to achieve immunological homoeostasis in the harshest of environments—namely, the intestine. Aberrant immune responses in the gut lead to the development of intestinal immune diseases such as colitis and food allergies[1,2]. Mast cells (MCs) are generally recognized as major effector cells of type 1 allergic diseases, as well as of inflammation, host defenses, innate and adaptive immune responses and homoeostatic responses[3–5]. Histological analyses of patients with, and murine models of, colitis have implicated the involvement of MCs in intestinal inflammation[4,6], but the factors responsible for MC activation are not fully understood.

Several lines of evidence have demonstrated that release of extracellular ATP and ADP from injured, dying or activated cells acts as a danger signal by modulating various cellular functions via the activation of P2 purinoceptors[7,8]. P2 purinoceptors comprise P2X ($P2X_{1-7}$) and P2Y receptors ($P2Y_{1, 2, 4, 6, 11-14}$). $P2X_{1-7}$ receptors are ATP-gated ion channels and specific for ATP, whereas P2Y receptors are G protein-coupled receptors that are specific for ADP, UTP and ATP[7,8].

Stimulation by ATP or ADP through the P2 purinoceptors of macrophages and dendritic cells (DCs) results in the production of inflammatory cytokines; this can lead to the development of asthma, contact hypersensitivity or graft-versus-host disease[9–11]. MCs also express several P2 purinoceptors and release histamine, cytokines and chemokines upon nucleotide stimulation[12]. Although MCs are thought to be involved in intestinal inflammation, it is unclear whether extracellular nucleotides are required for this process.

Here, we used a newly established anti-MC monoclonal antibody (mAb) to identify activated MCs and found that extracellular ATP mediates MC activation through P2X7 purinoceptors to initiate and amplify intestinal inflammation. Consequently, obstruction of the ATP-P2X7 purinoceptor cascade could be used to inhibit gut inflammatory diseases.

Results

Activated MCs in intestinal inflammation. Using a 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis model, we first examined whether MCs were involved in intestinal inflammation. To assess MC activation *in vivo*, we established an mAb (clone: 5A9) specific for CD63, a marker of activated MCs[13]. We confirmed that our anti-CD63 mAb was reactive specifically to MCs activated by immunoglobulin (Ig)E plus relevant allergen or a calcium ionophore, and not to naïve and CD63-knocked down MCs (Supplementary Fig. S1). In the colons of TNBS-treated mice, increased numbers of $CD63^+$-activated MCs were noted until day 3 post administration; the numbers then gradually decreased and reached a basal level on day 6 (Fig. 1a,b), indicating that MC activation was associated with the initiation phase of colitis development, as previously reported in a murine model and in patients with inflammatory bowel disease[6,14]. It has generally been accepted that the mechanistic basis of ulcerative colitis (UC) and Crohn's disease (CD) are different. Indeed, the pathogenic cytokines involved in the development of UC and CD are different[2] and the genetic polymorphisms specific for UC and CD are also different[15]. In addition, the cytokines required for the development of MCs differ between humans (stem cell factor) and mice [interleukin (IL)-3 and stem cell factor][4]. Thus, these different pathological environments may have led to differences in the requirement for, and involvement of, MCs in the development of inflammation. Therefore, we analysed MC numbers in both UD and CD patients, although we focused on the TNBS-induced colonic inflammation model. We detected increased numbers of MCs in the colons of patients with CD or UC (Fig. 1c,d). Thus, increased numbers of MCs in the colon is a characteristic of intestinal inflammation.

To directly show the involvement of MCs in the development of intestinal inflammation, we used MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. We confirmed that immunological and inflammatory symptoms induced by TNBS treatment were identical in $Kit^{W\text{-}sh/+}$ heterozygous and $Kit^{+/+}$ homozygous mice; however, inflammatory symptoms, such as body weight loss, massive inflammatory cell infiltration and colon shortening, were restored in $Kit^{W\text{-}sh/W\text{-}sh}$ mice but not in $Kit^{W\text{-}sh/+}$ heterozygous and $Kit^{+/+}$ homozygous mice (Fig. 1e–h). Similarly, our histological and immunological analyses revealed that destruction of the colonic epithelial layer and infiltration by inflammatory cells—especially neutrophils, which were stained neutral pink and had lobulated nuclei,—were reduced in $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 1f,h,i). Moreover, inflammatory signs were ameliorated in $Kit^{W\text{-}sh/W\text{-}sh}$ mice when we used other well-known inflammatory bowel disease models, such as the dextran sodium sulphate (DSS) colitis model (Fig. 2a–c). As the use of $Kit^{W\text{-}sh/W\text{-}sh}$ mice as an MC-deficient model is controversial[16,17], we also used the MC-specific enhancer-mediated toxin receptor-mediated conditional cell knockout (TRECK) system (Mas-TRECK mice)[18]. We confirmed that specific depletion of MCs ameliorated the inflammation in this DSS-induced colitis model (Fig. 2d–h). Our data indicate that activated MCs participate in the aggravation of intestinal inflammation.

Establishment of an inhibitory MC-specific mAb. IgE plus a relevant allergen induces MC activation; however, $Rag\text{-}1^{-/-}$ and $Tcr\beta^{-/-}\delta^{-/-}$ mice showed inflammatory responses comparable to those in TNBS-induced intestinal inflammation (Supplementary Fig. S2a–d)[19] and had increased numbers of $CD63^+$-activated MCs in their colons (Supplementary Fig. S2e), suggesting that T and B cells are not involved in MC activation during colitis. We also found no increase in CD63 expression on MCs after stimulation with IL-18 and IL-33, which are known to be involved in colitis (Supplementary Fig. S2f)[20,21].

We next tried to establish an anti-MC mAb that could ameliorate activated MC-mediated intestinal inflammation. We immunized rats with purified murine-activated colonic MCs, established hybridomas, performed flow cytometry to select hybridomas that produced mAbs that preferentially recognized colonic MCs and examined the hybridomas' ability to inhibit ovalbumin-induced food allergy[22] or TNBS-induced intestinal inflammation (Supplementary Fig. S3). Among 2,000 clones, we obtained an anti-MC mAb (designated clone 1F11; rat IgG2b) that was strongly reactive to colonic MCs (Fig. 3a; Supplementary Fig. S3). In addition to colonic MCs, 1F11 mAb bound efficiently to peritoneal cavity-, lung- and bone marrow (BM)-derived MCs, but not to skin MCs (Fig. 3a). When tested with other immunocompetent cells in the colon, 1F11 mAb was weakly reactive to some $CD3^+$ T cells, $CD11c^+$ DCs and $F4/80^+$ macrophages, but was not reactive to $Gr\text{-}1^+$ granulocytes, IgA^+ plasma cells or epithelial cells (ECs) (Fig. 3b).

To show the inhibitory function of 1F11 mAb in intestinal inflammation, mice were given 1F11 mAb ($0.5\,mg\,day^{-1}$ in a single dose) for 3 days, beginning 1 day before intrarectal administration of TNBS. 1F11 mAb treatment reduced the intestinal inflammation (Fig. 3c–g) and decreased the number of $CD63^+$-activated MCs in 1F11 mAb-treated mice (Fig. 3h).

Targeting P2X7 receptors reduces intestinal inflammation. Mass spectrometry analyses of immunoprecipitants of MC cell lysates with 1F11 mAb showed that the P2X7 purinoceptor is recognized by 1F11 mAb (Supplementary Fig. S4a). The specificity of 1F11 mAb for the P2X7 purinoceptor was confirmed by its specific reactivity to cells transfected with P2X7 receptors but not with other types of P2X receptor (for example, P2X1 and P2X4; Supplementary Fig. S4b). MCs derived from $P2x7^{-/-}$ mice, however, were not recognized by 1F11 mAb (Supplementary Fig. S4c). Western blot and flow cytometric analysis showed that, among the several variants of P2X7 purinoceptors[23], 1F11 mAb bound to variant a (full-length; Supplementary Fig. S4d,e). In contrast, variant c (possessing

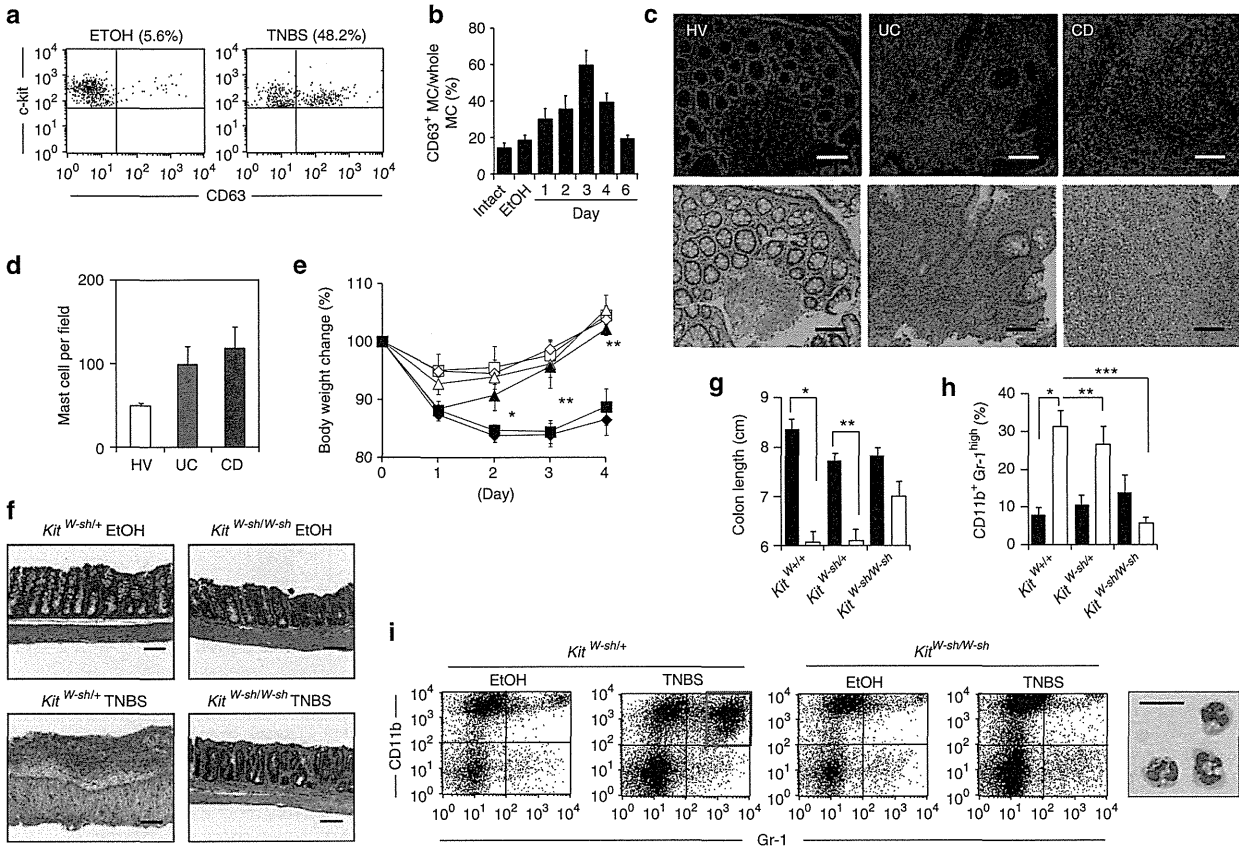

Figure 1 | Role of activated intestinal MCs in the development of intestinal inflammation. (**a**) CD63 expression on colonic MCs was examined with flow cytometry. Cells were gated on c-kit$^+$ and FcεRIα$^+$ cells. (**b**) The percentage of CD63$^+$ MCs in all c-kit$^+$ FcεRIα$^+$ MCs was determined with flow cytometry at various time points after TNBS administration ($n=3$ for day 6, $n=5$ for day 3, $n=7$ for intact, EtOH, day 1 and 2, $n=14$ for day 4). Control mice were analysed 4 days after EtOH administration (EtOH; $n=7$). Data are shown as means±s.e.m. (**c**) Colonic tissue sections from a healthy volunteer (HV) and UC and CD patients were stained with 4′,6-diamidino-2-phenyl indole (blue) and MC tryptase (red) or haematoxylin and eosin (H&E) (bottom). Scale bars, 100 μm. (**d**) Tryptase-positive MCs were counted in the fields of the tissue sections (four fields for each section). Data are means±s.e.m. ($n=6$). (**e**) Body weight changes were monitored after TNBS administration to *Kit* $^{W\text{-}sh/W\text{-}sh}$ MC-deficient mice (*Kit* $^{W\text{-}sh/W\text{-}sh}$ EtOH; open triangles: $n=4$, *Kit* $^{W\text{-}sh/W\text{-}sh}$ TNBS; closed triangles: $n=9$), *Kit* $^{+/+}$ control mice (*Kit* $^{+/+}$ EtOH; open diamonds: $n=4$, *Kit* $^{+/+}$ TNBS; closed diamonds: $n=13$) and *Kit* $^{W\text{-}sh/W+}$ control mice (*Kit* $^{W\text{-}sh/W+}$ EtOH; open squares: $n=4$, *Kit* $^{W\text{-}sh/W+}$ TNBS; closed squares: $n=11$). Data are shown as percentages of baseline weights and are means±s.e.m., $^*P<0.0001$ (two-tailed Student's *t*-test); $^{**}P=0.0024$ (two-tailed Student's *t*-test). (**f**) The colon was isolated 4 days after TNBS treatment for H&E staining. Data are representative of at least three independent experiments. Scale bars, 100 μm. (**g**) Colon length was measured 4 days after colitis induction. EtOH, closed column; TNBS, open column. $^*P<0.0001$ (two-tailed Student's *t*-test), $^{**}P=0.0024$ (two-tailed Student's *t*-test). Data are shown as means±s.e.m. (**h**) The percentage of CD11b$^+$ Gr-1^{high} cells in the colonic lamina propria was calculated, as measured with flow cytometry. EtOH, closed column; TNBS, open column. $^*P=0.0003$ (two-tailed Student's *t*-test), $^{**}P=0.0029$ (Welch's *t*-test) and $^{***}P<0.0001$ (Welch's *t*-test). Data are shown as means±s.e.m. (**i**) Colonic mononuclear cells were isolated 4 days after TNBS administration and stained with anti-CD11b and anti-Gr-1 antibodies. CD11b$^+$ Gr-1^{high} cells were sorted and then stained with May-Giemsa stain. Scale bar, 20 μm. Data are representative of three experiments.

the ATP-binding portion but lacking the C-terminal region) was detected by western blot, but its surface expression was not detected by flow cytometry because of its defect in extracellular expression (Supplementary Fig. S4d,e)[24]. In addition, neither western blot nor flow cytometry detected variant d (lacking the ATP-binding portion; Supplementary Fig. S4d,e). These data strongly suggest that 1F11 mAb recognizes P2X7 receptors, specifically the ATP-binding portion. We also confirmed that 1F11 mAb had similar reactivity to that of a commercially available anti-P2X7 mAb (clone: Hano43; Supplementary Fig. S4f,g).

To evaluate whether 1F11 mAb directly affects MCs during ATP-mediated activation, we treated MCs with ATP in the presence of 1F11 mAb *in vitro*. 1F11 mAb treatment reduced the number of CD63$^+$-activated MCs induced by ATP in a dose-dependent manner (Fig. 4a). High concentrations of extracellular ATP increased the cell permeability of the MCs[12]. Thus, uptake of Lucifer yellow was observed in ATP-stimulated MCs but was substantially impaired in 1F11 mAb-treated and *P2x7*$^{-/-}$ MCs (Fig. 4b,c).

As many cell types (MCs, T cells and DCs) express P2X7 receptors (Fig. 3b), we then asked whether the P2X7 receptors on MCs were responsible for the MC-mediated intestinal inflammation *in vivo* by analysing MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with *P2x7*$^{+/+}$ or *P2x7*$^{-/-}$ MCs. We confirmed that reconstituted MCs were present in the colon and maintained P2X7 expression (Supplementary Fig. S5). Like wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with *P2x7*$^{+/+}$ MCs showed severe inflammatory responses when treated with TNBS. However, these inflammatory responses were ameliorated when *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were reconstituted with *P2x7*$^{-/-}$ MCs; the amelioration included inhibition of neutrophil infiltration and MC activation (Figs 1 and 5a–f).

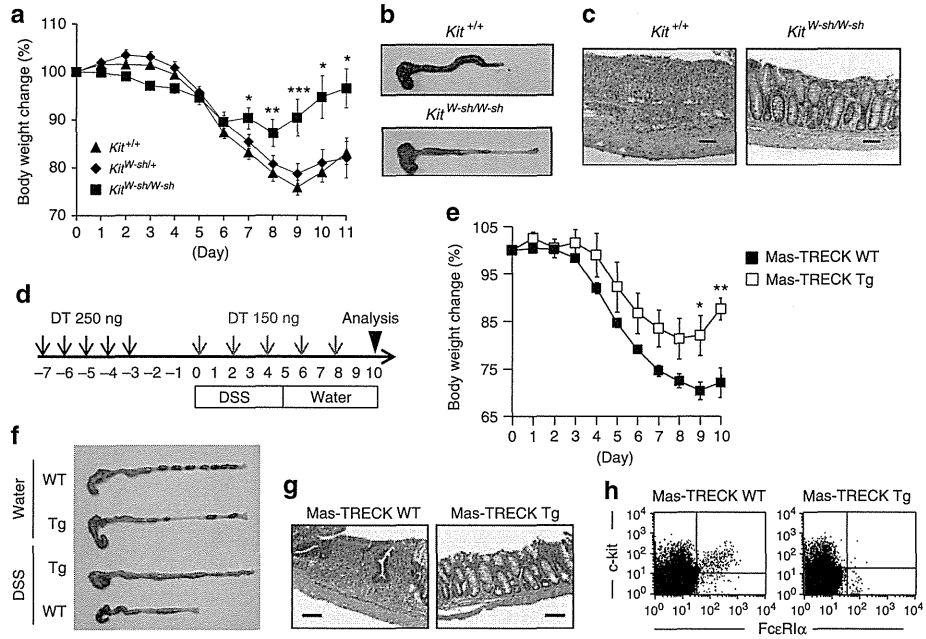

Figure 2 | Impaired DSS-induced colitis in MC-deficient mice. $Kit^{W\text{-}sh/W\text{-}sh}$ MC-deficient, $Kit^{+/+}$ control mice and Mas-TRECK transgenic (Tg) mice were subjected to DSS-induced colitis. (**a**) Body weight changes are shown as percentages of the baseline value and are means ± s.e.m. ($n = 22$ for $Kit^{+/+}$; $n = 25$ for $Kit^{W\text{-}sh/+}$; $n = 10$ for $Kit^{W\text{-}sh/W\text{-}sh}$). $^{*}P < 0.01$, $^{**}P = 0.0207$ and $^{***}P = 0.0004$ (two-tailed Student's *t*-test). (**b**,**c**) Eleven days after DSS treatment, colon tissue and haematoxylin and eosin (H&E)-stained tissue sections were examined. Data are representative of at least three independent experiments. (**d**) Mas-TRECK Tg mice and their wild-type (WT) littermates were subjected to DSS-induced colitis. For diphtheria toxin (DT) treatment, mice were injected intraperitoneally with 250 ng of DT for 5 consecutive days (black arrows) and then with 150 ng every other day (red allows). (**e**) Body weight changes are shown as percentages of the baseline value and are means ± s.e.m. ($n = 6$ for Tg; $n = 10$ for WT), $^{*}P = 0.0107$, $^{**}P = 0.0037$ (two-tailed Student's *t*-test). (**f**) Representative images of whole colons 10 days after DSS treatment. (**g**) Representative images of H&E staining. Scale bars, 100 μm. (**h**) Representative flow cytometric data of infiltrated c-kit^{+} FcεRIα^{+} MCs in the colon.

We next analysed whether the MCs in UC or CD patients expressed P2X7. Although increased number of MCs were observed in the colons of both UC and CD patients (Fig. 1c,d), P2X7 purinoceptors were expressed by the MCs in CD patients but not by those in UC patients or healthy volunteers (Fig. 5g,h). Thus, it is likely that P2X7 purinoceptor-mediated MC activation also occurs in the human colon, especially in CD patients.

To examine whether ATP was extracellularly released at high concentrations at inflammatory sites, we next measured ATP release from inflammatory colonic tissues. An elevated level of ATP release from the colon tissue was noted in TNBS-treated mice (Fig. 6a). In addition, intrarectal administration of non-hydrolyzable ATP (adenosine 5′-*O*-(3-thio) triphosphate and *O*-(4-benzoyl)benzoyl adenosine 5′-triphosphate) led to MC activation in the colonic tissue, similar to the effect of TNBS treatment (Fig. 6b). In contrast, intrarectal administration of other P2Y receptor agonists did not increase colonic MC activation (Fig. 6b). These findings indicate that inflammatory stimuli induce the extracellular release of ATP, which in turn leads to P2X7-dependent MC activation in the colon and subsequent exacerbation of intestinal inflammation.

P2X7 signalling activates the caspase-1 inflammasome to induce the production of IL-1β and IL-18 (ref. 25). IL-1β production is also mediated by MC proteases, such as chymases[26]. We therefore examined whether MCs produced IL-1β via P2X7 receptor activation, and if so whether this production was caspase-1-dependent. IL-1β production was decreased when P2X7-deficient MCs were stimulated with ATP, whereas substantial amounts of IL-1β were produced in caspase-1-deficient MCs (Supplementary Fig. S6), indicating that IL-1β production was P2X7-dependent but caspase-1-independent. In line with this finding, body weight changes were noted in $Kit^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with *caspase-1*$^{-/-}$ MCs (Fig. 5a). These results suggest that MC-dependent inflammation through P2X7 purinoceptors is not dependent on caspase-1-mediated IL-1β or IL-18 production.

An autocrine loop of ATP conversion mediates MC activation. In addition to ATP, other nucleotides (for example, extracellular ADP) act as signals to induce inflammatory responses[27]. We confirmed that MCs are activated by high concentrations of ADP and ATP (Fig. 7a,b). Extracellular ATP is hydrolysed by ectonucleoside triphosphate diphosphohydrolases (CD39) to ADP and AMP; it is then further hydrolysed by ecto-5′-nucleotidase (CD73) to adenosine, which has anti-inflammatory functions[27]. Colonic MCs expressed CD39 but not CD73 (Supplementary Fig. S7a,b), indicating that MCs can convert ATP to ADP but not to adenosine. We therefore examined the involvement of ADP-reactive P2Y purinoceptors and found that P2Y1 and P2Y12 were highly expressed on colonic MCs (Fig. 7c). However, inhibitors of P2Y1 and P2Y12 receptors, as well as knockdown of the P2Y12 receptor, had no effect on the induction of CD63^{+}-activated MCs (Fig. 7d,e; Supplementary Fig. S8a). Similarly, intestinal inflammation, as well as activation of colonic MCs, was unaffected in clopidogrel (a P2Y12 receptor inhibitor)-treated mice (Supplementary Fig. S8b–d). These data indicate that although P2Y1 and P2Y12 were expressed on MCs neither P2Y1 nor P2Y12 purinoceptors mediate ADP-dependent CD63^{+} MC induction.

It is generally accepted that P2X7 purinoceptors specifically recognize ATP[7], but we found that they were also involved in ADP-mediated MC activation. Indeed, no activation was noted in *P2x7*$^{-/-}$ MCs when they were stimulated with ADP (Fig. 7f), leading us to hypothesize that ADP promotes ATP release from MCs and their subsequent stimulation. To test this hypothesis, we measured the expression of pannexin-1, connexin 43 and connexin 32, which

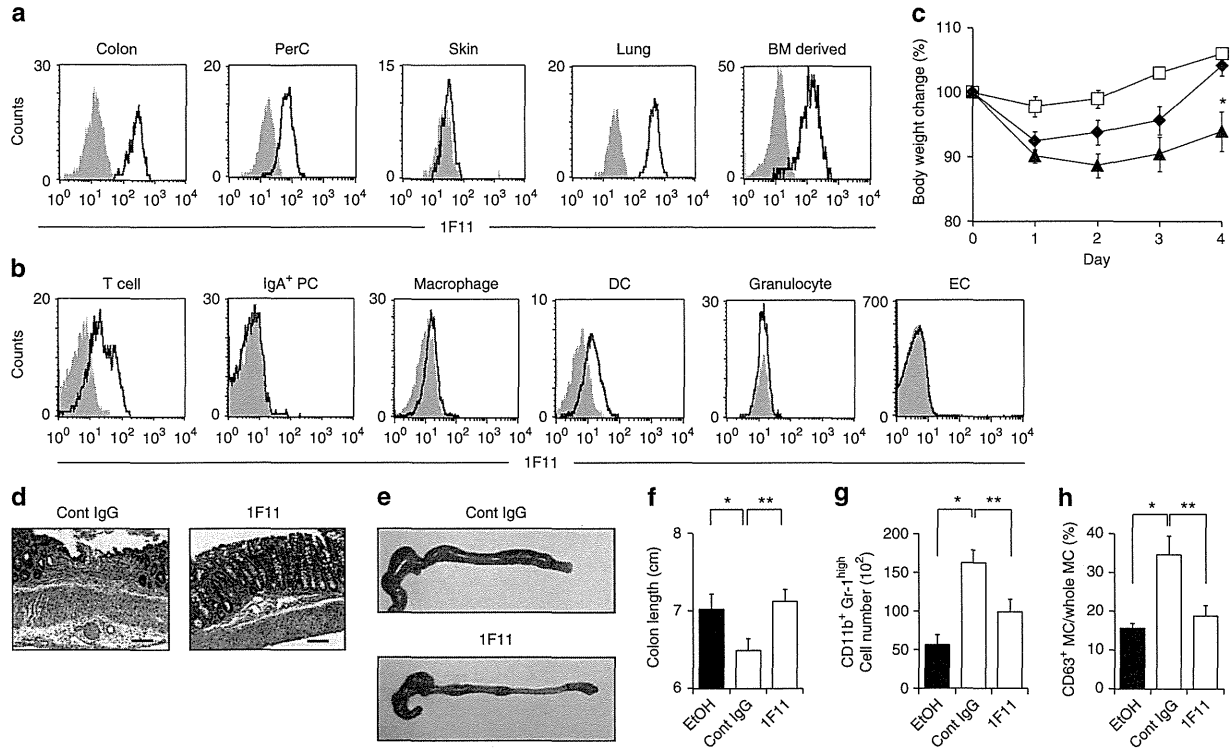

Figure 3 | Amelioration of colitis by treatment with intestinal MC-reactive 1F11 mAb. (**a**) MCs in the colonic lamina propria, peritoneal cavity (PerC), skin and lung, as well as BM-derived MCs, were stained with 1F11 mAb. Control staining with rat IgG2b is shown in grey. (**b**) Cells were isolated from colonic lamina propria and epithelium. $CD3^+$ T cells, IgA^+ plasma cells (PCs), $F4/80^+$ macrophages, $CD11c^+$ DCs, $Gr1^+$ granulocytes and ECs were gated and their reactivity to 1F11 mAb examined. Control staining with rat IgG2b is shown in grey. (**c**) C57BL/6 mice were treated with TNBS and their body weights were monitored for 4 days; 0.5 mg of 1F11 or the control mAb was intraperitoneally administered. Data from 9 (EtOH; open squares), 19 (TNBS with control mAb; closed triangles) and 12 (TNBS with 1F11 mAb; closed diamonds) mice. $^*P = 0.0066$ (Welch's *t*-test). Data are shown as percentages of baseline weights and are means±s.e.m. (**d**,**e**) Representative images of haematoxylin and eosin staining and colon tissue from 1F11 mAb-treated mice. Scale bars, 100 μm. (**f**) Colon length was measured 4 days after TNBS administration. $^*P = 0.0445$; $^{**}P = 0.0073$ (two-tailed Student's *t*-test). (**g**) Neutrophils ($CD11b^+$ $Gr\text{-}1^{high}$) were quantified as percentages and numbers of cells. Data are shown as means±s.e.m. ($n = 6$), $^*P < 0.0001$, $^{**}P = 0.0047$ (two-tailed Student's *t*-test). (**h**) Percentage of $CD63^+$ MCs in all c-kit^+ FcεRIα$^+$ MCs was determined with flow cytometry. Data are shown as means±s.e.m. ($n = 6$) $^*P = 0.0202$; $^{**}P = 0.0284$ (two-tailed Student's *t*-test).

are ATP-releasing hemichannels, during cell activation[28,29]. The hemichannels were rarely expressed on the colonic MCs (Fig. 7g), and no inhibitory effect was observed when the MCs were treated with ADP in the presence of hemichannel inhibitors (flufenamic acid and carbenoxolone). However, cell activation was inhibited by P2X7 antagonists [oxidized ATP (OxATP), pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate] (Fig. 7h). To further exclude the possibility that ADP triggers ATP release, we stimulated MCs with another P2Y ligand (UTP); we found that UTP did not induce MC activation (Fig. 7b).

We then tested whether ADP was converted to ATP by ATP-converting enzymes such as ecto-adenylate kinase, ATP synthase and nucleoside diphosphokinase[30]. To test the involvement of these enzymes, we used inhibitors of ecto-adenylate kinase (diadenosine pentaphosphate; AD2P5), ATP synthase (oligomycin; oligo) and nucleoside diphosphokinase (UDP), and we found that inhibition of ecto-adenylate kinase and ATP synthase, but not nucleoside diphosphokinase, reduced ADP- as well as ATP-dependent MC activation (Fig. 7h,i). Neither AD2P5 nor oligo inhibited MC activation induced by the crosslinking of IgE with relevant allergen (Fig. 7i). Among the adenylate kinases, adenylate kinase 1 (AK1) and AK2 were expressed in colonic MCs, and the expression of AK2 was much higher than that of AK1 (Supplementary Fig. S9a). As with AD2P5 treatment, knockdown of AK2, but not AK1, led to the inhibition of both ADP- and ATP-mediated MC activation (Supplementary Fig. S9b). These results indicate that P2X7 purinoceptors have an important role in the activation of MCs by ATP, including ATP derived from ADP by the action of ecto-enzymes such as ATP synthase and AK2.

Neutrophil infiltration by MC-derived mediators. Evaluation of MC activation on the basis of CD63 expression is an important criterion[13]; however, degranulation is not absolutely associated with cytokine production[31]. Therefore, we measured MC production of an array of inflammatory cytokine, chemokine and lipid mediators to additionally elucidate the role of P2X7 purinoceptor-mediated MC activation in the development of intestinal inflammation. Stimulation of MCs with ATP induced the production of inflammatory cytokines such as IL-6, tumour necrosis factor (TNF)α and oncostatin M[32]; this induction was not observed in $P2x7^{-/-}$ MCs or in wild-type MCs treated with 1F11 mAb (Fig. 8a,b).

We showed that neutrophil infiltration into the colon was mediated by MC activation (Fig. 1h,i), and a previous study suggested that neutrophil infiltration is a potential target in colitis treatment[33]. Consistent with these findings, ATP stimulation induced MCs, but not $P2x7^{-/-}$ MCs, to produce leukotrienes (LTs; LT C4/D4/E4), which are associated with the translocation of 5-lipoxygenase (5-LO) into the nucleus—an important step for LT synthesis in MCs[34] (Fig. 8c,d). Also, chemokine gene array analysis demonstrated that