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ADAM10 is a principal 'sheddase' of the low-affinity immunoglobulin E receptor CD23

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CD23, the low-affinity immunoglobulin E receptor, is an important modulator of the allergic response and of diseases such as rheumatoid arthritis. The proteolytic release of CD23 from cells is considered a key event in the allergic response. Here we used loss-of-function and gain-of-function experiments with cells lacking or overexpressing candidate CD23-releasing enzymes (ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19 and ADAM33), ADAM-knockout mice and a selective inhibitor to identify ADAM10 as the main CD23-releasing enzyme *in vivo*. Our findings provide a likely target for the treatment of allergic reactions and set the stage for further studies of the involvement of ADAM10 in CD23-dependent pathologies.

The low-affinity immunoglobulin E (IgE) receptor CD23 is an important mediator of the allergic response and can function to enhance antigen presentation of IgE antigen complexes[1]. CD23 is a type II integral membrane protein containing a C-terminal lectin head, a leucine zipper, a transmembrane domain and a short cytoplasmic tail. Studies of knockout and transgenic animals have shown that CD23 negatively regulates IgE production. A soluble form of CD23 (sCD23) is released from the plasma membrane after membrane-proximal cleavage. This sCD23 still retains low-affinity IgE-binding activity and interacts with human CD21 on the B cell surface. In the human immune system, sCD23 enhances IgE production by peripheral blood mononuclear cells stimulated with interleukin 4 (IL-4) and hydrocortisone, whereas antibody (anti-CD23) directed against the lectin domain of CD23 inhibits IgE production in the same model[2]. CD23-deficient mice on a 'low responder' background have enhanced IgE production after immunization with antigen plus alum[3], whereas mice overexpressing surface CD23 demonstrate substantial inhibition of allergic responses[4]. In addition to its effects on allergic disease, sCD23 has been linked to the activation of macrophages through interaction with CD11b-CD18 on the cell surface, resulting in the release of proinflammatory mediators and the onset of inflammatory disease[5,6]. Mice treated with anti-CD23 have considerable amelioration of inflammatory disease[7], and CD23-deficient mice have delayed onset of arthritis in a collagen-induced model[8]. Moreover, sCD23 concentrations are increased in inflamed joints in rheumatoid arthritis[9,10]. Finally, membrane CD23 is increased in B cell chronic lymphocytic leukemia, and increased concentrations of sCD23 in serum are a negative prognostic indicator of patient survival[11,12].

With such potentially harmful consequences resulting from the cleavage of CD23, the CD23-releasing enzyme, or CD23 'sheddase', is a likely target for the design of drugs to treat allergic diseases and possibly autoimmune diseases such as rheumatoid arthritis. Early work with inhibitors of various enzyme classes indicated involvement of a hydroxamate-sensitive metalloprotease of approximately 62 kilodaltons (kDa) in CD23 shedding in a variety of cell types[13]. That finding raised the possibility that the CD23 sheddase is a member of the ADAM family of membrane-anchored metalloproteases, which have also been linked to a variety of other ectodomain-shedding events, such as the release of tumor necrosis factor (TNF), epidermal growth factor receptor ligands and several other membrane proteins[14,15]. Further information on the nature of the CD23 sheddase has emerged from a study comparing the effect of various hydroxamates on the cleavage of CD23 and TNF. The inhibitor profile of hydroxamates that block TNF shedding is distinct from those responsible for CD23 shedding, suggesting that the main CD23 sheddase is distinct from the TNF convertase (ADAM17)[16]. Additional attempts to characterize the CD23 sheddase have focused on peptide-cleavage assays using purified active catalytic domains of ADAM8, ADAM15 and ADAM28. Those experiments have shown that the catalytic

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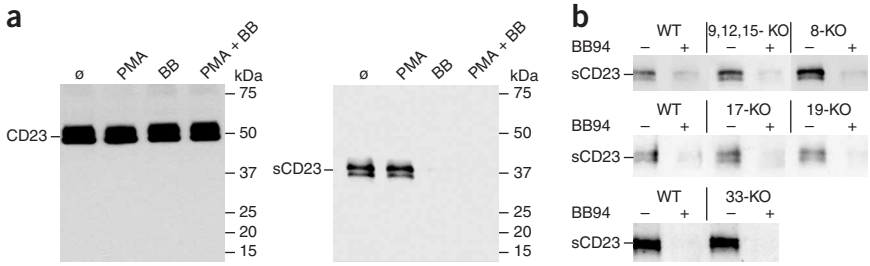

Figure 1 Shedding of CD23 in wild-type and ADAM-deficient MEFs. Primary MEFs from wild-type or ADAM-deficient mice were transfected with CD23 cDNA and were treated with PMA and/or BB94; CD23 was immunoprecipitated with antibody 30X (anti–human CD23) and was analyzed by immunoblot with anti–V5 tag (binds to a C-terminal V5 tag in expressed CD23). (**a**) Supernatants (right) or cell lysates (left) of wild-type primary MEFs, collected after 3 h of no treatment (ø) or culture in the presence of 25 ng/ml of PMA (PMA), 1 μM BB94 (BB) or 25 ng/ml of PMA plus 1 μM BB94 (PMA + BB). (**b**) Shedding of sCD23 by primary MEFs from wild-type mice (WT), *Adam9*$^{-/-}$*Adam12*$^{-/-}$*Adam15*$^{-/-}$ mice (9,12,15–KO), *Adam8*$^{-/-}$ mice (8-KO), *Adam17*$^{-/-}$ mice (17-KO), *Adam19*$^{-/-}$ mice (19-KO) or *Adam33*$^{-/-}$ mice (33-KO) in the presence (+) or absence (–) of 1 μM BB94. All experiments were done at least three times with similar results.

domains of all three ADAM proteases can cleave a peptide corresponding to the membrane-proximal cleavage site of CD23 (ref. 17). In addition, overexpression of ADAM8 leads to an increase in CD23 shedding in cell-based assays. However, the expression pattern of ADAM8 is restricted[18], raising the possibility that enzymes other than ADAM8 are more relevant for CD23 shedding *in vivo*.

To evaluate the function of various ADAM proteases in CD23 shedding, we did loss-of-function experiments with cells isolated from mice lacking various widely expressed and catalytically active ADAM proteases that can be considered candidate CD23 sheddases. In addition, we did gain-of-function experiments by overexpressing various ADAM proteases with CD23 to analyze which of those enzymes can in principle release CD23 from cells. Moreover, we stimulated CD23 shedding in mice lacking ADAM8, ADAM9, ADAM12 or ADAM15 by injecting an antibody to the CD23 stalk region to assess the involvement of those ADAM proteases in CD23 shedding *in vivo*. This antibody (19G5) enhances the susceptibility of CD23 to cleavage by unfolding the CD23 trimer so that the cleavage site is more readily accessible to the CD23 'sheddase'[19]. Finally, we used a selective hydroxamate inhibitor to evaluate the inhibitor profile of CD23 shedding in mouse and human primary B cells. Our results support the hypothesis that ADAM10 is critical for CD23 shedding and challenge the idea of an essential and chief function for ADAM8 or other ADAM proteases in CD23 shedding.

RESULTS

Loss-of-function studies with ADAM-deficient cells

To evaluate the contribution of various ADAM proteases to CD23 shedding in cell-based assays, we transiently expressed human CD23 in primary embryonic fibroblasts from wild-type mice and mice lacking various widely expressed and catalytically active ADAM proteases (ADAM8, ADAM9, ADAM12, ADAM15, ADAM17, ADAM19 and ADAM33). We assessed shedding of the CD23 ectodomain by immunoblot analysis. In wild-type mouse embryonic fibroblasts (MEFs) expressing membrane-anchored 45-kDa CD23, both the 37-kDa and 33-kDa forms of human sCD23 were released into the supernatant in the absence of treatment (**Fig. 1a**). Thus, the molecular masses of full-length CD23 expressed in MEFs (45 kDa) and of the two soluble forms released from those cells (37 kDa and 33 kDa) are similar to those described before for other cell types, including the human B lymphoblastoid cell line RPMI 8866 and Chinese hamster ovary cells[13]. Shedding of both forms of sCD23 was not stimulated substantially by 25 ng/ml of phorbol-12-myristate-13-acetate (PMA) in wild-type MEFs (**Fig. 1a**), similar to the activity of the CD23 sheddase in B cell chronic lymphatic leukemia cells, in that shedding was not stimulated by PMA[20]. Moreover, release of sCD23 in unstimulated and PMA-stimulated cells was efficiently blocked by the hydroxamic acid–type metalloprotease inhibitor batimastat (BB94; 1 μM; **Fig. 1a**). We did similar experiments with primary MEFs from mice lacking ADAM8 (ref. 18), triple-knockout mice lacking ADAM9, ADAM12 and ADAM15 (ref. 21) and mice lacking ADAM17 (ref. 22), ADAM19 (ref. 23) or ADAM33 (prepared by S.U., unpublished data); in these cells, CD23 shedding was similar to that of wild-type control cells (**Fig. 1b**). We used immunoblot analysis to confirm that the expression of CD23 in cell lysates was similar in wild-type and ADAM-deficient cells (data not shown). In all cases, the shedding was reduced considerably by the addition of 1 μM BB94 (**Fig. 1b**). These results demonstrated that none of the ADAM proteases listed above are essential for the BB94-sensitive component of constitutive CD23 shedding in MEFs.

ADAM10 is a constitutively active enzyme that is inhibited by BB94 and is stimulated only weakly by the addition of PMA[21]. The weak response of the CD23 sheddase to PMA stimulation in MEFs thus raised the possibility that ADAM10 is responsible for that activity. Moreover, shedding of CD23 in wild-type MEFs was stimulated considerably by the addition of the calcium ionophore ionomycin (**Fig. 2a**), consistent with involvement of ADAM10 in this process[24]. Constitutive shedding of CD23 was not visible here (**Fig. 2a**), as the conditioned medium was collected after 30 min of ionomycin treatment, whereas conditioned medium was otherwise collected after 3 h for detection of constitutive shedding. The ionomycin-induced shedding of CD23 was inhibited substantially by 1 μM BB94 (**Fig. 2a**) and by 2 μM GI254023X, a pharmacological inhibitor with selectivity for ADAM10 versus ADAM17 at this concentration[25], although a small amount of 35-kDa sCD23 was still released in the presence of ionomycin and either inhibitor (**Fig. 2a**).

To further explore the possibility that ADAM10 was responsible for shedding CD23, we evaluated the shedding of CD23 in immortalized MEFs from wild-type, *Adam10*$^{+/-}$ or *Adam10*$^{-/-}$ mice. When we introduced CD23 into immortalized wild-type control cells, sCD23 was released into the supernatant, and its shedding was blocked by 1 μM BB94 (**Fig. 2b**). Release of sCD23 from *Adam10*$^{+/-}$ cells was similar to that of wild-type cells and was also blocked by 1 μM BB94 (**Fig. 2b**). In *Adam10*$^{-/-}$ cells, there was no release of sCD23 in the presence or absence of 1 μM BB94 (**Fig. 2b**), even though CD23 expression in the cell lysates was similar for all three cell types (data not shown). When we cotransfected *Adam10*$^{-/-}$ cells with mouse ADAM10 cDNA and human CD23 cDNA, shedding of sCD23 was restored (**Fig. 2b**). These findings were consistent with the idea of a chief function for ADAM10 in the shedding of sCD23 from MEFs.

Gain-of-function studies with overexpressed ADAM proteases

ADAM8, ADAM15 and ADAM28 have been linked to CD23 shedding because their purified protease domains are able to cleave a peptide

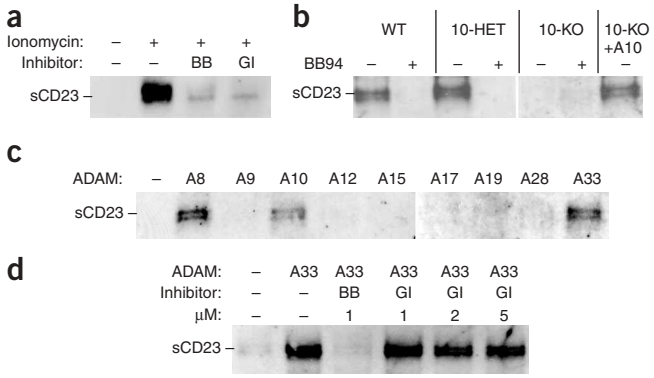

Figure 2 Characterization of candidate CD23 sheddases in wild-type and *Adam10*$^{-/-}$ MEFs. (**a**) Immunoblot analysis of CD23 shed from wild-type MEFs in the presence (+) or absence (–) of 2.5 μM ionomycin and 1 μM BB94 (BB), 2 μM GI254023X (GI) or no inhibitor (–). (**b**) The generation of sCD23 in the presence (+) or absence (–) of BB94 or in the presence of cotransfected mouse ADAM10 cDNA (+ A10) by immortalized wild-type MEFs (WT), *Adam10*$^{+/-}$ MEFs (10-HET) or *Adam10*$^{-/-}$ MEFs (10-KO). (**c**) Analysis of the ability of ADAM8 (A8), ADAM9 (A9), ADAM10 (A10), ADAM12 (A12), ADAM15 (A15), ADAM17 (A17), ADAM19 (A19), ADAM28 (A28) and ADAM33 (A33) to restore the shedding of sCD23 when overexpressed in immortalized *Adam10*$^{-/-}$ cells. (**d**) Shedding of sCD23 in *Adam10*$^{-/-}$ cells in the presence (A33) or absence (–) of cotransfected ADAM33, with or without 1 μm BB94 or 1 μM, 2 μM or 5 μM GI254023X. Data are representative of at least three experiments with similar results.

corresponding to the cleavage site of CD23 *in vitro*[17]. In addition, overexpression of ADAM8 increases CD23 shedding from cells and ADAM8 interacts with CD23 (ref. 17). Moreover, ADAM33 has been reported as a candidate CD23 sheddase[26]. To corroborate those results and to test which other ADAM proteases were able to cleave human CD23 in cell-based assays when overexpressed, we individually coexpressed ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, ADAM28 or ADAM33 with human CD23 in *Adam10*$^{-/-}$ cells. We chose *Adam10*$^{-/-}$ cells for these gain-of-function assays, as any contribution by one or more other ADAM proteases to CD23 shedding should be more readily detectable in the absence of that CD23 sheddase.

These experiments confirmed that overexpressed ADAM8, which we included as a positive control, was able to restore CD23 shedding in *Adam10*$^{-/-}$ cells (**Fig. 2c**). In addition, ADAM33 released sCD23 when overexpressed (**Fig. 2c,d**). The ADAM33-dependent release of CD23 was blocked by 1 μM BB94 but not by 1 μM, 2 μM or 5 μM GI254023X (**Fig. 2d**), at which concentration GI254023X blocks ADAM10-dependent shedding of the chemokine CX3CL1 (ref. 27) and of epidermal growth factor (EGF; discussed below). However, in identical conditions, we found no evidence of a contribution of ADAM9, ADAM12, ADAM15, ADAM17, ADAM19 or ADAM28 to the shedding of CD23 (**Fig. 2c**). In parallel experiments, ADAM9 and ADAM12 were able to cleave EGF, ADAM17 was able to cleave transforming growth factor-α, and ADAM19 cleaved the cytokine TRANCE (also called OPGL), which served as a positive control for the activity of these ADAM proteases in cell-based assays[23,28] (K. Horiuchi and C.P.B., data not shown). We confirmed the expression, proper maturation and prodomain removal of ADAM15 and ADAM28 by immunoblot analysis (data not shown), as there is no positive control for the catalytic activity of these two ADAM proteases in cell-based assays at present. These gain-of-function experiments confirmed that ADAM8 and ADAM33 are able to cleave CD23, raising the possibility that they could contribute to CD23 shedding *in vivo*, such as in cells or tissues in which either ADAM8 or ADAM33 is or both are highly expressed. However, no positive evidence of the involvement of ADAM9, ADAM12, ADAM15, ADAM17, ADAM19 and ADAM28 emerged from either the loss-of-function or gain-of-function studies in MEFs, challenging the idea of a substantial contribution of those ADAM proteases to CD23 shedding in mice.

Shedding of endogenous CD23 in ADAM-deficient mice

To assess whether ADAM8, ADAM9, ADAM12 or ADAM15 was critical for CD23 shedding *in vivo*, we injected *Adam8*$^{-/-}$ mice, *Adam9*$^{-/-}$*Adam15*$^{-/-}$ double-knockout mice and *Adam9*$^{-/-}$*Adam12*$^{-/-}$*Adam15*$^{-/-}$ triple-knockout mice with the 19G5 antibody to the stalk of CD23, which enhances the susceptibility of CD23 to cleavage compared with use of an isotype control antibody[19]. Preliminary experiments indicated that one injection of 1 mg 19G5 induced sCD23 release by 100- to 150-fold over either an isotype control antibody or PBS vehicle control and resulted in substantial loss of B cell surface CD23 (J.W.F and D.H.C, unpublished observation). We injected ADAM-deficient mice or wild-type control mice with 19G5 or PBS vehicle control, then, 3 d later, collected spleens and serum and assessed cell surface expression of CD23 on splenocytes by flow cytometry. In all cases, membrane-associated CD23 was equally low in 19G5-treated mice, indicating that the antibody was able to effectively enhance CD23 shedding even in the absence of ADAM8

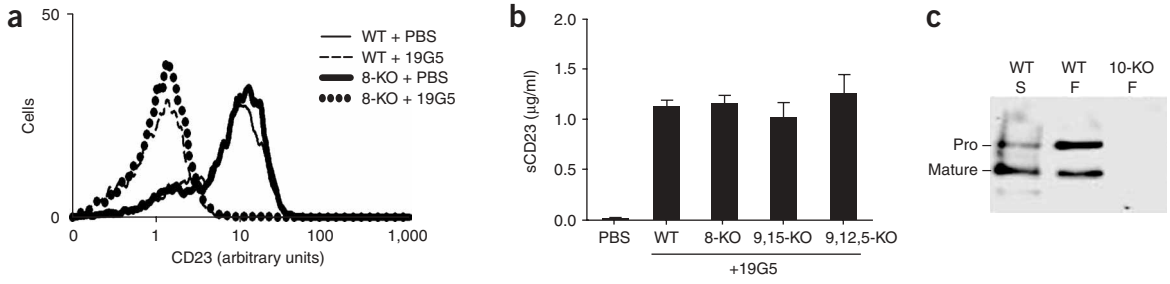

Figure 3 Nondefective CD23 shedding in *Adam8*$^{-/-}$, *Adam9*$^{-/-}$*Adam15*$^{-/-}$ or *Adam9*$^{-/-}$*Adam12*$^{-/-}$*Adam15*$^{-/-}$ mice. ADAM-deficient mice ($n = 5$ per strain) or wild-type control mice ($n = 5$) were injected with PBS or 1 mg 19G5; after 3 d, serum and spleens were collected for analysis. (**a**) Flow cytometry of CD23 expression by splenocytes, gated on B cells. Data are representative of two independent experiments with similar results. (**b**) ELISA of sCD23 concentration in serum. Data are representative of three independent experiments (error bars, s.e.m.). (**c**) Immunoblot of ADAM10 expression on wild-type splenocytes (WT S), wild-type fibroblasts (WT F) and *Adam10*$^{-/-}$ fibroblasts (10-KO F). Left margin, 'pro-form' and mature form of ADAM10. Data are representative of at least three independent experiments.

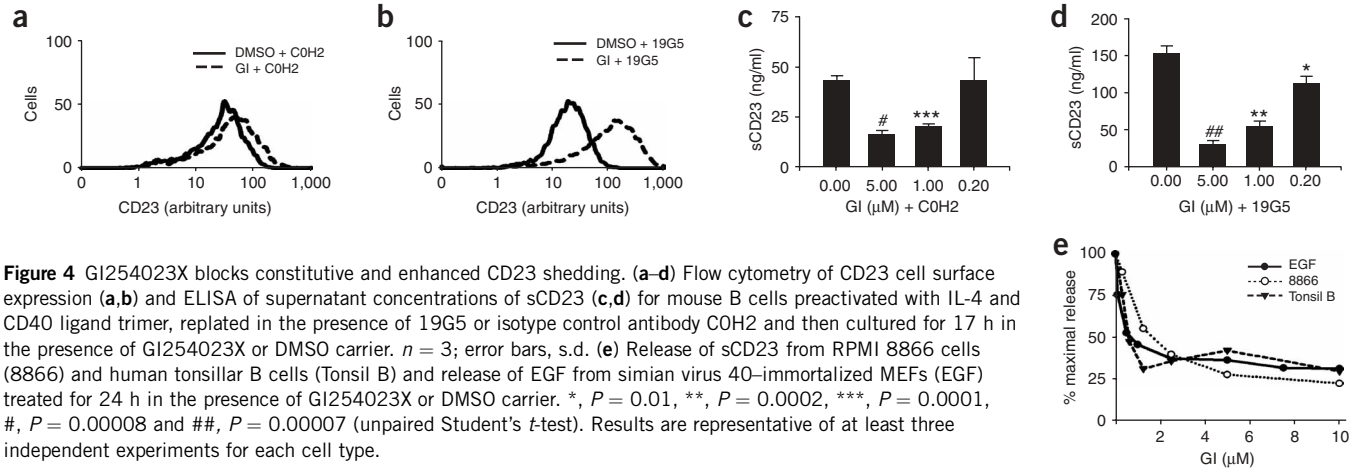

Figure 4 GI254023X blocks constitutive and enhanced CD23 shedding. (**a**–**d**) Flow cytometry of CD23 cell surface expression (**a**,**b**) and ELISA of supernatant concentrations of sCD23 (**c**,**d**) for mouse B cells preactivated with IL-4 and CD40 ligand trimer, replated in the presence of 19G5 or isotype control antibody C0H2 and then cultured for 17 h in the presence of GI254023X or DMSO carrier. $n = 3$; error bars, s.d. (**e**) Release of sCD23 from RPMI 8866 cells (8866) and human tonsillar B cells (Tonsil B) and release of EGF from simian virus 40–immortalized MEFs (EGF) treated for 24 h in the presence of GI254023X or DMSO carrier. *, $P = 0.01$, **, $P = 0.0002$, ***, $P = 0.0001$, #, $P = 0.00008$ and ##, $P = 0.00007$ (unpaired Student's *t*-test). Results are representative of at least three independent experiments for each cell type.

(**Fig. 3a**), ADAM9 and ADAM15, or ADAM9, ADAM12 and ADAM15 (data not shown). CD23 expression can be upregulated by activation with CD40 ligand trimer and IL-4 (ref. 29). To determine if the ADAM-deficient mice had any defects in their ability to induce CD23 expression, we next cultured splenocytes from the mice injected with 19G5 or PBS with CD40 ligand trimer and IL-4. After that treatment, CD23 concentrations on splenic B cells were similar to those on wild-type control cells, confirming an equal capacity for CD23 induction in *Adam8*$^{-/-}$ mice and *Adam9*$^{-/-}$*Adam15*$^{-/-}$ mice as well as *Adam9*$^{-/-}$*Adam12*$^{-/-}$*Adam15*$^{-/-}$ mice (data not shown). As an additional means of monitoring CD23 shedding *in vivo*, we assayed by enzyme-linked immunosorbent assay (ELISA) the serum sCD23 concentrations of ADAM-deficient mice and wild-type control mice that had been injected with 19G5 or PBS. After injection with 19G5, serum sCD23 titers were increased in *Adam8*$^{-/-}$, *Adam9*$^{-/-}$*Adam15*$^{-/-}$ and *Adam9*$^{-/-}$*Adam12*$^{-/-}$*Adam15*$^{-/-}$ mice to a degree similar to that in wild-type control mice (**Fig 3b**). The sCD23 concentrations in PBS-injected mice were much lower than those in antibody-treated mice (less than 10 ng/ml; **Fig. 3b**). These findings challenged the idea of critical involvement of ADAM8, ADAM9, ADAM12 or ADAM15 in 19G5-stimulated release of CD23 into mouse serum.

Effect of an ADAM10-selective inhibitor on primary B cells

As we found no evidence for involvement of ADAM8, ADAM9, ADAM12 or ADAM15 in CD23 shedding in mice *in vivo* and had ruled out ADAM17, ADAM19 and ADAM28 based on the studies reported above, ADAM10 and ADAM33 were still viable candidates for being the main CD23 sheddase in mice. ADAM10 was critically involved in CD23 shedding in both gain-of-function and loss-of-function studies in MEFs, whereas ADAM33 was able to elicit shedding of CD23 only in the gain-of-function experiment (**Fig. 2d**) but was not required in the loss-of-function experiment (**Fig. 1b**). Immunoblot analysis of mouse splenocytes confirmed expression of ADAM10 in these cells (**Fig. 3c**), whereas ADAM33 is not expressed in splenocytes and B cells[30]. The ADAM10-selective hydroxamate GI254023X can distinguish between the activity of ADAM10 and ADAM33 at concentrations between 1 μM and 5 μM (the inhibitor profile of GI254023X for ADAM10-dependent shedding has been determined in cell-based assays[25,27]; discussed below). GI254023X is thus an informative tool for testing the relevance of ADAM10 for CD23 shedding in primary cells, especially as ADAM10-deficient mice die by day 9.5 *in utero*, so CD23 shedding cannot be evaluated in B cells from *Adam10*$^{-/-}$ mice[31].

To test the hypothesis that ADAM10 is also critical for CD23 shedding in primary mouse B cells, we isolated those cells from CD23-transgenic mice[4] and preactivated them with IL-4 and CD40 ligand trimer (described above). CD23-transgenic mice express more CD23 on lymphocytes than do wild-type mice, which facilitated detection of CD23 cleavage *in vitro*. We replated the cells after the activation period in the presence of 19G5 or an isotype control antibody (C0H2) and assessed the ability of GI254023X to block CD23 processing both at the cell surface (**Fig. 4a**,**b**) and in the supernatant (**Fig. 4c**,**d**) after additional culture. GI254023X blocked both constitutive CD23 cleavage (**Fig. 4a**,**c**) and 19G5-enhanced CD23 cleavage (**Fig. 4b**,**d**), and cell surface expression of CD23 was moderately increased in the presence of GI254023X, with the greatest enhancement at a concentration of 5 μM (**Fig. 4a**,**b**). These findings indicated that both constitutive and enhanced CD23 cleavage was reduced in the presence of GI254023X at a concentration (1 μM) at which it does not affect the ability of ADAM33 to shed CD23 in *Adam10*$^{-/-}$ MEFs (**Fig. 2d**). However, small amounts of sCD23 shedding still occurred, indicating minor background processing. Analysis of sCD23 concentrations after culture of human tonsillar B cells or of the strongly CD23^{+} lymphoblastoid cell line RPMI 8866 in the presence of GI254023X for up to 24 h showed dose-dependent inhibition of sCD23 release. The inhibitor profile of sCD23 release from those cells closely resembled the dose-dependent inhibition of EGF shedding in MEFs (**Fig. 4e**), which depends on ADAM10 (ref. 21). Only a residual amount of processing remained in the presence of the highest dose of GI254023X in both cases. That inhibitor profile for GI254023X provides further support for the idea of ADAM10 as the main constitutive and 19G5-stimulated CD23 sheddase in a human B cell line and in both the mouse and human *ex vivo* assays with primary cells.

DISCUSSION

The sheddase for the low-affinity IgE receptor CD23 is considered a likely target for the design of drugs to treat asthma and allergic responses as well as rheumatoid arthritis. The identification of ADAM10 as the main CD23 sheddase is a critical step, and a prerequisite for further studies of its physiological relevance as a CD23 sheddase in diseases such as asthma. Given the interest in blocking IgE synthesis as a treatment for type I allergy, the identification of ADAM10 as the CD23 sheddase could lead to new approaches for allergy treatment. Transgenic mice overexpressing CD23 had much less IgE production, suggesting that increasing cell-associated CD23 by blocking its shedding might have a similar effect and thus be beneficial

for patients with asthma[4]. That finding is further supported by the observation that wild-type mice as well as mice with severe combined immunodeficiency that have been reconstituted with human peripheral blood leukocytes have reduced IgE when treated with metalloprotease inhibitors[32,33]. The metalloprotease inhibitor BB94, which has been used in wild-type mice[32], is known to inhibit ADAM10 (ref. 21) as well as other metalloprotease activities[34].

However, a caveat is that ADAM10 is a pleiotropic enzyme with several critical functions in development and presumably also in diseases such as cancer. In flies, mice and worms, ADAM10 is required for Notch signaling[31,35,36], and it also is thought to be involved in several other physiological and pathological processes, such as cleaving amyloid precursor protein[37] or N-cadherin[38]. The usefulness of ADAM10 as a drug target will depend mainly on how critical it is for adult homeostasis, which in turn will affect possible side effects of systemic treatment. However, at least for asthma, the use of inhalers might allow local drug delivery and help circumvent deleterious side effects of blocking ADAM10. Studies of mice with conditional knockout of ADAM10 as well as with selective pharmacological inhibitors and different routes of drug delivery will be necessary to address those issues. If blocking ADAM10 results in adverse side effects, an alternative could be to devise new methods to directly block CD23 shedding, such as with function-blocking antibodies or other reagents that block CD23 shedding without affecting other functions of its sheddase. Notably, the identification of a monoclonal antibody that induces shedding of CD23 demonstrates that it is also possible to specifically affect the release of a membrane protein without directly activating the responsible protease. Although that antibody would presumably not be useful for treatment of asthma, the ability to specifically induce shedding of a receptor might be beneficial in other situations, such as removing cell surface molecules with deleterious functions in disease (for example, Her2 in breast cancer).

In summary, our data have provided experimental evidence for the involvement of ADAM10 in the production of sCD23 in MEFs and primary mouse and primary human B cells. Both loss-of-function and gain-of-function experiments failed to demonstrate involvement of ADAM9, ADAM12, ADAM15, ADAM17, ADAM19 or ADAM28 in CD23 shedding. Moreover, CD23 shedding was not detectably affected in mice lacking ADAM8, and a pharmacological inhibitor blocked CD23 shedding in primary B cells at a concentration at which it does not block ADAM33, suggesting that those two are irrelevant for CD23 shedding *in vivo*. It will now be necessary to study mice with conditional knockout of ADAM10 and/or mice transgenic for dominant negative ADAM10 to determine the involvement of ADAM10 in CD23 shedding in mice and thus in immunoreceptor-mediated disease.

METHODS

Animals and reagents. *Adam8*$^{-/-}$ mice[18], *Adam9*$^{-/-}$*Adam15*$^{-/-}$ mice, *Adam9*$^{-/-}$*Adam12*$^{-/-}$*Adam15*$^{-/-}$ mice[21], CD23-transgenic mice[4] and C57BL/6 littermate mice were maintained in accredited animal facilities (at the Hospital for Special Surgery and the Virginia Commonwealth University) according to the guidelines of the American Veterinary association, and all experiments were approved by the respective Institutional Animal Care and Use Committees. PMA was purchased from Sigma. Concanavalin A and protein A–Sepharose beads were obtained from Pharmacia, and ionomycin was purchased from Calbiochem. Restriction enzymes and other enzymes were obtained from Roche Biochemicals. BB94 and GI254023X[25,27] were also used.

Antibodies and cDNA. The cDNA encoding human CD23 and a C-terminal V5 tag and six-histidine tag in the pcDNA3 expression plasmid was obtained from Invitrogen. The expression vectors (pcDNA3; Invitrogen) for mouse ADAM proteases have been described[39]. Antibodies to human CD23 (30X) were generated as described (**Supplementary Fig. 1** online). Antibodies to the cytoplasmic domain of human ADAM10 were from Chemicon. Horseradish peroxidase–labeled secondary antibodies were purchased from Promega. The preparation and purification[40] of antibody 19G5 to mouse CD23 stalk was as described[19]. Antibodies for flow cytometry, rat anti–mouse CD23 (clone B3B4) and rat anti–mouse B220 (clone RA3-6B2), were obtained from BD PharMingen. The human B cell purification reagents anti-IgD (clone IA6-2) and anti–fluorescein isothiocyanate microbeads were purchased from BD PharMingen and Miltenyi Biotec, respectively. Mouse CD40 ligand trimer and anti–isoleucine zipper[41] (clone M15) were obtained from Amgen. Human IL-4 was purchased from R&D Systems and cells producing anti-CD40 (G28.5) were obtained from American Type Culture Collection.

Cell culture, transfection and immunoblot analysis. Primary MEFs were generated from wild-type or ADAM-deficient embryos at embryonic day 13.5 and were cultured as described[21,42]. *Adam10*$^{+/-}$ and *Adam10*$^{-/-}$ fibroblast cell lines derived from embryos at embryonic day 9.5 have been described[31]. Transfection, shedding assays and immunoblot analysis were done as described[21,42] with the following exceptions. Cells were incubated for 3 h in the presence of inhibitors or for 30 min in the presence of ionomycin. Supernatants for CD23 analysis were clarified by centrifugation for 30 min at 16,000*g* in a tabletop centrifuge and were precipitated for 1 h at 24 °C with 30X antibody and protein A–Sepharose beads. Reducing SDS-PAGE sample loading buffer (10 mM dithiothreitol) was added immediately, followed by heating to 95 °C, and the released proteins were separated by SDS-PAGE and were transferred to nitrocellulose membranes. Ponceau S staining confirmed equivalent transfer of proteins to nitrocellulose for all lanes. Membranes were then probed with horseradish peroxidase–labeled anti–V5 tag (Invitrogen) and bound antibodies were visualized by enhanced chemiluminescence (Amersham).

For analysis of the effect of GI254023X on the shedding of EGF from simian virus 40–immortalized wild-type MEFs, these cells were transfected with alkaline phosphatase–tagged EGF and were cultured for 1 h with various concentrations of GI254023X, and release of EGF was monitored by colorimetric detection of the activity of soluble alkaline phosphatase in the culture supernatant as described[21].

Human tonsils were obtained from routine tonsillectomies (protocol 03237, Human Tonsils as a Source of B Lymphocytes, approved by the Virginia Commonwealth University Institutional Review Board), and IgD$^+$ B cells were isolated by standard magnetic-activated cell sorting techniques (Miltenyi Biotec) as described[43]; purity averaged at least 95% by flow cytometry. Human B cells were cultured in complete media supplemented with 10 ng/ml of recombinant human IL-4 and 1 μg/ml of anti–human CD40 (G28.5). RPMI 8866 cells were maintained as described[44]. Mouse splenic B cells were purified by negative selection by means of antibody-labeled T cell depletion with guinea pig complement combined with isolation of total cells by Percoll gradients[19]. Labeled antibodies were used for flow cytometry, and propidium iodide was added for exclusion of dead cells. Stained cells were analyzed with a Cytomics FC500 Flow Cytometer and data were analyzed with CXP software (Beckman Coulter).

Analysis of shedding of mouse and human CD23 *in vitro* and *in vivo*. Purified mouse B cells (1×10^6 cells/ml) were cultured for 24 h in 24-well tissue culture plates (Costar) with 25 ng/ml of CD40 ligand trimer, 0.1 μg/ml of M15 antibody and 10,000 U/ml of IL-4. Cells were then washed and were replated with fresh activation agents plus either 100 μg/ml of antibody 19G5 or isotype control antibody (C0H2) in the presence or absence (dimethyl sulfoxide carrier only) of GI254023X. After 17 h, cell surface CD23 was analyzed by flow cytometry or sCD23 in supernatants was measured by ELISA. Purified human tonsillar B cells were used in similar experiments, except G28.5 (anti-CD40; 1 μg/ml) and 10 ng/ml of recombinant human IL-4 were used for the initial culture (48 h), and washed cells were replated for an additional 24 h with activation agents with or without GI254023X. For RPMI 8866 cells, 1×10^6 cells per ml were cultured for 24 h with 0.3125–10 μM GI254023X. For both, sCD23 concentrations were then measured by ELISA; the ELISAs for mouse and human sCD23 have been described[43,45]. For *in vivo* analyses, five wild-type mice or *Adam8*$^{-/-}$, *Adam9*$^{-/-}$*Adam15*$^{-/-}$ *or Adam9*$^{-/-}$*Adam12*$^{-/-}$*Adam15*$^{-/-}$ mice of mixed genetic background (C57BL/6–129Sv) were injected intraperitoneally with 200 μl PBS or with 1 mg of antibody 19G5 in 200 μl PBS. Then, 3 d after

injection, blood was obtained from mice by cardiac puncture and the concentration of sCD23 in the collected serum was then analyzed by ELISA. Spleens were collected and single-cell suspensions were made using frosted glass slides. Splenocytes were incubated with 2.4G2 (anti–Fcγ receptor[46]) and were stained for 1 h at 4 °C with fluorescein isothiocyanate–B3B4 (anti–lectin CD23) and allophycocyanin-B220 (anti-CD45R). Surface expression of CD23 was analyzed by flow cytometry (described above).

Statistics. Unpaired Student's *t*-tests were used to calculate *P* values.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

G.W., J.W.F., J.S, D.H.C and C.P.B. contributed to the experimental design, data analysis, critical discussions and manuscript preparation; G.W. did the experiments in **Figures 1** and **2**, the immunoblot in **Figure 3c** and the titration of the GI254023X inhibitor in **Figure 4e** for EGF in MEFs; J.W.F prepared and purified antibody 19G5 and did the experiments in **Figures 3a,b** and **4a–d**; J.S. tested how GI254023X affects CD23 shedding from RPMI 8866 cells and primary human tonsillar B cells (**Fig. 4e**); S.M. and N.B. provided antibody 30X, which both had characterized before, and critical information for its use in **Figures 1** and **2**; ADAM-deficient mice were supplied by A.J.P.D. (*Adam8*$^{-/-}$), A.S.-F. (*Adam12*$^{-/-}$) and R.A.B. (*Adam17*$^{-/-}$); *Adam10*$^{-/-}$, *Adam10*$^{+/-}$, wild-type control cells and mouse ADAM10 cDNA were from P.S. and D.H.; *Adam33*$^{-/-}$ cells and wild-type controls were from S.U.; S.S. injected wild-type and ADAM-deficient mice with antibody 19G5, isolated serum and collected spleens for the experiments in **Figure 3a,b**; A.L. and J.D.B provided GI254023X and BB94; J.D.B. provided information and discussions, especially at beginning; D.H.C and C.P.B supervised this study; and all authors contributed to the final stages of manuscript preparation.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Immunology* website for details).

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1. Heyman, B. Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annu. Rev. Immunol.* **18**, 709–737 (2000).
2. Pene, J. *et al.* Interleukin 5 enhances interleukin 4-induced IgE production by normal human B cells. The role of soluble CD23 antigen. *Eur. J. Immunol.* **18**, 929–935 (1988).
3. Yu, P., Kosco-Vilbois, M., Richards, M., Kohler, G. & Lamers, M.C. Negative feedback regulation of IgE synthesis by murine CD23. *Nature* **369**, 753–756 (1994).
4. Payet, M.E., Woodward, E.C. & Conrad, D.H. Humoral response suppression observed with CD23 transgenics. *J. Immunol.* **163**, 217–223 (1999).
5. Lecoanet-Henchoz, S. *et al.* CD23 regulates monocyte activation through a novel interaction with the adhesion molecules CD11b-CD18 and CD11c-CD18. *Immunity* **3**, 119–125 (1995).
6. Lecoanet-Henchoz, S. *et al.* Mouse CD23 regulates monocyte activation through an interaction with the adhesion molecule CD11b/CD18. *Eur. J. Immunol.* **27**, 2290–2294 (1997).
7. Plater-Zyberk, C. & Bonnefoy, J.Y. Marked amelioration of established collagen-induced arthritis by treatment with antibodies to CD23 *in vivo*. *Nat. Med.* **1**, 781–785 (1995).
8. Kleinau, S., Martinsson, P., Gustavsson, S. & Heyman, B. Importance of CD23 for collagen-induced arthritis: delayed onset and reduced severity in CD23-deficient mice. *J. Immunol.* **162**, 4266–4270 (1999).
9. Huissoon, A.P., Emery, P., Bacon, P.A., Gordon, J. & Salmon, M. Increased expression of CD23 in rheumatoid synovitis. *Scand. J. Rheumatol.* **29**, 154–159 (2000).
10. Ribbens, C. *et al.* Increased synovial fluid levels of soluble CD23 are associated with an erosive status in rheumatoid arthritis (RA). *Clin. Exp. Immunol.* **120**, 194–199 (2000).
11. Molica, S. *et al.* Cellular expression and serum circulating levels of CD23 in B cell chronic lymphocytic leukemia. Implications for prognosis. *Haematologica* **81**, 428–433 (1996).
12. Sarfati, M. *et al.* Prognostic importance of serum soluble CD23 level in chronic lymphocytic leukemia. *Blood* **88**, 4259–4264 (1996).
13. Marolewski, A.E. *et al.* CD23 (FcεRII) release from cell membranes is mediated by a membrane-bound metalloprotease. *Biochem. J.* **333**, 573–579 (1998).
14. Seals, D.F. & Courtneidge, S.A. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes Dev.* **17**, 7–30 (2003).
15. Blobel, C.P. ADAMs: key players in EGFR-signaling, development and disease. *Nat. Rev. Mol. Cell Biol.* **6**, 32–43 (2005).
16. Parkin, E.T. *et al.* Structure-activity relationship of hydroxamate-based inhibitors on the secretases that cleave the amyloid precursor protein, angiotensin converting enzyme, CD23, and pro-tumor necrosis factor-α. *Biochemistry* **41**, 4972–4981 (2002).
17. Fourie, A.M., Coles, F., Moreno, V. & Karlsson, L. Catalytic activity of ADAM8, ADAM15, and MDC-L (ADAM28) on synthetic peptide substrates and in ectodomain cleavage of CD23. *J. Biol. Chem.* **278**, 30469–30477 (2003).
18. Kelly, K. *et al.* Metalloprotese-disintegrin ADAM8: Expression analysis and targeted deletion in mice. *Dev. Dyn.* **232**, 221–231 (2005).
19. Kilmon, M.A., Shelburne, A.E., Chan-Li, Y., Holmes, K.L. & Conrad, D.H. CD23 trimers are preassociated on the cell surface even in the absence of its ligand, IgE. *J. Immunol.* **172**, 1065–1073 (2004).
20. Gu, B., Bendall, L.J. & Wiley, J.S. Adenosine triphosphate-induced shedding of CD23 and L-selectin (CD62L) from lymphocytes is mediated by the same receptor but different metalloproteases. *Blood* **92**, 946–951 (1998).
21. Sahin, U. *et al.* Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR-ligands. *J. Cell Biol.* **164**, 769–779 (2004).
22. Peschon, J.J. *et al.* An essential role for ectodomain shedding in mammalian development. *Science* **282**, 1281–1284 (1998).
23. Zhou, H.M. *et al.* Essential role for ADAM19 in cardiovascular morphogenesis. *Mol. Cell. Biol.* **24**, 96–104 (2004).
24. Nagano, O. *et al.* Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca^{2+} influx and PKC activation. *J. Cell Biol.* **165**, 893–902 (2004).
25. Ludwig, A. *et al.* Metalloproteinase inhibitors for the disintegrin-like metalloproteinases ADAM10 and ADAM17 that differentially block constitutive and phorbol ester-inducible shedding of cell surface molecules. *Comb. Chem. High Throughput Screen.* **8**, 161–171 (2005).
26. Rosenwasser, L.J. & Meng, J. Anti-CD23. *Clin. Rev. Allergy Immunol.* **29**, 61–72 (2005).
27. Hundhausen, C. *et al.* The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. *Blood* **102**, 1186–1195 (2003).
28. Peduto, L., Reuter, V.E., Shaffer, D.R., Scher, H.I. & Blobel, C.P. Critical function for ADAM9 in mouse prostate cancer. *Cancer Res.* **65**, 9312–9319 (2005).
29. Keegan, A.D., Snapper, C.M., Van Dusen, R., Paul, W.E. & Conrad, D.H. Super-induction of the murine B cell Fc epsilon RII by T helper cell clones. Role of IL-4. *J. Immunol.* **142**, 3868–3874 (1989).
30. Umland, S.P. *et al.* Human ADAM33 messenger RNA expression profile and post-transcriptional regulation. *Am. J. Respir. Cell Mol. Biol.* **29**, 571–582 (2003).
31. Hartmann, D. *et al.* The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for α-secretase activity in fibroblasts. *Hum. Mol. Genet.* **11**, 2615–2624 (2002).
32. Christie, G. *et al.* IgE secretion is attenuated by an inhibitor of proteolytic processing of CD23 (Fc epsilonRII). *Eur. J. Immunol.* **27**, 3228–3235 (1997).
33. Mayer, R.J. *et al.* Inhibition of CD23 processing correlates with inhibition of IL-4-stimulated IgE production in human PBL and hu-PBL-reconstituted SCID mice. *Clin. Exp. Allergy* **30**, 719–727 (2000).
34. Roghani, M. *et al.* Metalloprotease-disintegrin MDC9: intracellular maturation and catalytic activity. *J. Biol. Chem.* **274**, 3531–3540 (1999).
35. Rooke, J., Pan, D., Xu, T. & Rubin, G.M. KUZ, a conserved metalloprotease-disintegrin protein with two roles in *Drosophila* neurogenesis. *Science* **273**, 1227–1230 (1996).
36. Wen, C., Metzstein, M.M. & Greenwald, I. SUP-17, a *Caenorhabditis elegans* ADAM protein related to *Drosophila* KUZBANIAN, and its role in LIN-12/NOTCH signaling. *Development* **124**, 4759–4767 (1997).
37. Lammich, S. *et al.* Constitutive and regulated α-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc. Natl. Acad. Sci. USA* **96**, 3922–3927 (1999).
38. Reiss, K. *et al.* ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. *EMBO J.* **24**, 742–752 (2005).
39. Horiuchi, K., Zhou, H.M., Kelly, K., Manova, K. & Blobel, C.P. Evaluation of the contributions of ADAMs 9, 12, 15, 17, and 19 to heart development and ectodomain shedding of neuregulins β1 and β2. *Dev. Biol.* **283**, 459–471 (2005).
40. Schwartz, W. *et al.* Application of chemically-stable Immunoglobulin-selective sorbents: harvest and purification of antibodies with resolution of aggregate. *BioProcessing J.* **3**, 53–62 (2004).
41. Fanslow, W.C. *et al.* Structural characteristics of CD40 ligand that determine biological function. *Semin. Immunol.* **6**, 267–278 (1994).
42. Weskamp, G. *et al.* Mice lacking the metalloprotease-disintegrin MDC9 (ADAM9) have no evident major abnormalities during development or adult life. *Mol. Cell. Biol.* **22**, 1537–1544 (2002).
43. Caven, T.H. *et al.* IL-21 dependent IgE production in human and mouse in vitro culture systems is cell density and cell division dependent and is augmented by IL-10. *Cell. Immunol.* **238**, 123–134 (2005).
44. Conrad, D.H. & Peterson, L.H. The murine lymphocyte receptor for IgE. I. Isolation and characterization of the murine B cell Fcε receptor and comparison with Fcε receptors from rat and human. *J. Immunol.* **132**, 796–803 (1984).
45. Kilmon, M.A. *et al.* Regulation of IgE production requires oligomerization of CD23. *J. Immunol.* **167**, 3139–3145 (2001).
46. Unkeless, J.C. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* **150**, 580–596 (1979).