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The emergence of ADAM10 as a regulator of lymphocyte development and autoimmunity

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Abstract

Proteolytic processing of transmembrane receptors and ligands can have a dramatic impact on cell signaling processes and subsequent cellular responses, including activation and differentiation. A member of the disintegrin and metalloproteinase family, ADAM10, has emerged as a prominent regulator of numerous receptors and ligands, including Notch and CD23. Here, we review studies resulting from the recent generation of ADAM10 conditional knockout mice which revealed a critical role for ADAM10 in Notch-dependent lymphocyte development. Additionally, we discuss results of numerous *in vitro* and *ex vivo* studies indicating that ADAM10 regulates the production of multiple secreted factors that contribute to autoimmune reactions.

Keywords

a disintegrin and metalloproteinase 10; Notch; CD23; *Kuzbanian*; lymphocyte development; autoimmunity

1. Introduction

Given that receptor:ligand binding events are critical for intercellular signaling, proteolytic processing of transmembrane receptors and ligands can have a dramatic impact on signaling-mediated cellular responses. Proteolysis of eukaryotic proteins is performed by members of the metzincin superfamily, including matrix metalloproteinases (MMPs), disintegrin and metalloproteinases (ADAMs), and ADAM-thromospondins (ADAM-TS). Although all active metzincin proteases contain zinc-binding motifs within their protease domains, the presence of a transmembrane domain is characteristic of the ADAMs family. Thus, studies examining the processing of receptors and ligands expressed on cell surfaces have focused on ADAMs, which perform ectodomain shedding as well as regulated intramembrane proteolysis (RIP) of transmembrane proteins. Shedding extracellular domains of membrane-anchored proteins releases soluble fragments into extracellular space. This event can down-regulate signaling events that require transmembrane receptor expression or activate paracrine signaling by soluble products derived from ADAM substrates, such as soluble CD23 (sCD23) and tumor necrosis factor- α (TNF- α). Although

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ectodomain shedding is thought to occur constitutively, RIP requires the binding of ligands expressed on adjacent cells. Numerous receptors including Notch and CD44 require regulated proteolysis of the receptor:ligand complex to initiate the release of receptor intracellular domains (ICD) that translocate to the nucleus and alter gene expression. Mutations in the negative regulatory region (NRR) of ADAM substrates can cause ligand-independent intramembrane proteolysis, resulting in excessive ICD signaling and disease states, discussed below (Blobel, 2005; Crawford *et al.*, 2009; Seals and Courtneidge, 2003).

The prototypical ADAM contains an inhibitory pro-domain, a highly conserved metalloprotease domain, a disintegrin domain conferring substrate specificity, a cysteine-rich region, a transmembrane portion, and a cytoplasmic tail capable of binding SH3 domains (Seals and Courtneidge, 2003). Although 38 ADAMs have been identified to date, only a subset of members, including ADAMs 8, 9, 10, 12, 15, 17, and 33, contain the conserved zinc-binding consensus motif that confers proteolytic activity to the protease domain. As a result of *in vitro*-based assays utilizing pharmacologic inhibitors and various ADAM-deficient murine embryonic fibroblasts (MEFs), ADAM10 has emerged as an important mediator of ectodomain shedding and RIP of numerous substrates, including amyloid plaque precursor (APP), Ephrins, cadherins, chemokines, Notch receptors, Delta-1, CD23, Lag-3, FasL, and CD44. Proteolytic processing of many of these substrates contributes to the pathogenesis of multiple disease states, including cancer and inflammation (Blobel, 2005; Crawford *et al.*, 2009). In particular, the role of ADAM10-mediated APP processing and its effect on Alzheimer's disease have been extensively studied and are reviewed elsewhere (Postina, 2008). Here, we discuss recent studies that highlight the physiologic role of ADAM10-mediated cleavage events in lymphocyte development and inflammation related to autoimmunity. Additionally, we review reports describing novel functions of ADAM10, and the regulation of proteolytic activity.

2. The rate-limiting protease in Notch RIP

2.1 A historical perspective

Since its discovery, it was evident that ADAM10 and its *drosophila* homolog, *Kuzbanian* (*kuz*), perform critical roles in developmental pathways. *Kuz* was initially identified by Rooke *et al.* in 1996, who generated *kuz*⁻ embryos that revealed its essential role in lateral inhibition required for development of peripheral and central nervous systems. Cloning and sequence analysis of *kuz* demonstrated the presence of disintegrin and metalloproteinase domains (Rooke *et al.*, 1996). Database searches identified a mammalian homolog (43% amino acid identity), bovine metalloprotease (BMP, later named ADAM10 (Wolfsberg *et al.*, 1995)) that was isolated from brain myelin and shown to cleave myelin basic protein *in vitro* (Howard and Glynn, 1995). By overexpressing dominant negative (DN) mutants of *kuz* that lack the protease domain in *drosophila* and *Xenopus*, Pan *et al.* demonstrated that the defect in lateral inhibition is due to the requirement for *kuz* to initiate RIP-mediated signaling through the *Notch* receptor (Pan and Rubin, 1997). This finding was supported by Sotillos *et al.* However, the authors acknowledged phenotypic differences between *kuz* and *Notch* mutant flies and concluded that *Notch* could also be processed in a *kuz*-independent manner (Sotillos *et al.*, 1997).

In mammalian cells, signaling through Notch receptors critically regulates cell fate processes of numerous cell types, including murine embryos. Following receptor ligation of Notch ligands, including Delta-like (Dll) 1-4 and Jagged 1-2, expressed on adjacent cells, Notch signaling is initiated by ADAM-mediated proteolysis of the extracellular domain of the Notch receptor that is endocytosed by the ligand-expressing cell. This cleavage event, termed the S2 cleavage, produces a substrate for the γ -secretase complex to perform S3 cleavage and release the Notch intracellular domain (NICD) from the cell membrane. This

results in NICD translocation to the nucleus, where it complexes with the transcription factor RBP-J κ , and induces transcription of Notch target genes, including Hairy enhancer of split (Hes1), Hes5, and Deltex-1 (Figure 1).

Although numerous regulators of Notch signaling have been identified, the identity of the metalloprotease that initiates RIP-mediated Notch signaling in mammalian cells was highly controversial (Kopan and Ilagan, 2009). Initially, two independent groups concluded that ADAM17 (TNF- α converting enzyme, TACE) initiates signaling by performing S2 cleavage of the Notch1 receptor (Brou *et al.*, 2000; Mumm *et al.*, 2000). Loading cell membrane fragments from Notch-transfected HeLa cells onto RED-TSK columns resulted in co-elution of Notch receptor cleavage products with ADAM17, rather than ADAM10. Additionally, results of an *in vitro* cleavage assay and a monocytic cell differentiation experiment demonstrated ADAM17's ability to cleave truncated forms of Notch1 and direct Notch-dependent monocyte differentiation (Brou *et al.*, 2000). Mumm *et al.* supported this finding by demonstrating Notch cleavage in transfected MEFs derived from ADAM10-null embryos (Mumm *et al.*, 2000). As a result, ADAM17 is often referenced in the literature as the relevant proteinase that initiates Notch signaling (Radtke *et al.*, 2004). Although these findings were in direct contrast to studies of *kuz* in *drosophila*, the authors could not rule out a role for ADAM10 in Notch cleavage, and suggested that ADAM10 and ADAM17 may be functionally redundant *in vivo*. This conclusion was supported by later *in vitro* studies demonstrating that ADAM10 can cleave many ADAM17 substrates from ADAM17^{-/-} MEFs (Le Gall *et al.*, 2009), while ADAM17 can also cleave numerous ADAM10 substrates (Hinkle *et al.*, 2004; Sahin *et al.*, 2004). However, a critical role for ADAM10 in Notch activation re-emerged following the generation of ADAM10-deficient mouse embryos that displayed many features also observed in nonviable Notch1^{-/-} embryos (Hartmann *et al.*, 2002; Swiatek *et al.*, 1994). In contrast, embryonic loss of ADAM17 did not result in a Notch phenotype (Peschon *et al.*, 1998). Following these observations, two groups utilizing ADAM10^{-/-} MEFs recently reported that while multiple proteases can perform ligand-independent proteolysis of Notch1, ADAM10 is required for ligand-dependent cleavage of Notch (Bozkulak E.C. and Weinmaster, 2009; van Tetering G. *et al.*, 2009). Thus, ADAM10 may play a more critical role in Notch signaling than earlier *in vitro* studies predicted. However, *in utero* lethality of ADAM10-null embryos at day E9.5 had limited the examination of ADAM10-mediated cleavage events in the development of other cell types, including lymphocytes.

2.2 T cell development

The impact of Notch signaling in T cell development has been thoroughly examined, and is reviewed elsewhere (Tanigaki and Honjo, 2007). Briefly, Notch1 signaling is essential for the development of thymocyte precursors. This is best illustrated by the presence of thymic B cells in mice that lack Notch1 expression in common lymphoid progenitors (CLPs) (Wilson *et al.*, 2001). Additionally, enforced Notch1 signaling in bone marrow progenitors expressing the constitutively active NICD promotes T cell fate (Pui *et al.*, 1999). In fact, human mutations in the NRR surrounding the S2 cleavage site of Notch1 results in ligand-independent proteolysis and excessive Notch1 activation, ultimately causing T cell acute lymphocytic leukemia (T-ALL). This mutation accounts for approximately 50 percent of all T-ALL cases (Kopan and Ilagan, 2009). Multiple groups have reported effective use of γ -secretase inhibitors (GSIs) in limiting T cell development *in vitro* and in mouse models of T-ALL (Real *et al.*, 2009; Sanda *et al.*, 2010). However, GSIs cause gastrointestinal disease and must be coupled with potent anti-inflammatory drugs, such as dexamethasone, in T-ALL mouse models (Real *et al.*, 2009). Thus, there has been great interest in elucidating the role of ADAMs in Notch1-mediated thymocyte development.

Manilay et al. circumvented the limitation of ADAM10^{-/-} embryo lethality by generating transgenic mice that overexpress the dominant negative form of ADAM10 (dnKuz) under control of the T cell-specific promoter, lck(Manilay *et al.*, 2005). dnKuz expression caused a partial block in thymocyte development between the double negative (DN) and double positive (DP) stages. This corresponded to decreased TCR β expression and pre-mature down-regulation of CD25. Although these findings were also observed in Notch1-deficient thymocytes(Wolfer *et al.*, 2002), there were discrete differences in thymocyte development and gene expression between these mice. dnKuz mice have reduced levels of DN thymocytes and $\gamma\delta$ T cells, whereas conditional Notch1-deficient mice do not, suggesting that ADAM10 may regulate early thymocyte development by processing other substrates in addition to Notch1. Furthermore, expression of Notch target genes, Hes1 and Deltex-1, was only moderately decreased compared to non-transgenic mice. However, this could be due to the persistence of endogenous ADAM10 expression in dnKuz mice. Interestingly, enforced expression of the predominant Notch ligand, Dll1, rescued thymocyte development in dnKuz mice in a non-cell autonomous manner. This indicated that ADAM10 may regulate thymocyte development by processing Dll1 expressed on adjacent cells. This possibility is supported by studies demonstrating that ADAM10 also cleaves Dll1 in transfected MEFs(Six *et al.*, 2003). However, another study demonstrated that Dll1 is a substrate of multiple other ADAMs(Dyczynska *et al.*, 2007). Nevertheless, the impaired development of dnKuz thymocytes is also consistent with the conclusion that dnKuz impairs ADAM10-dependent processing of the Notch1 receptor.

Further examination of ADAM10's role in thymocyte development awaited the production of ADAM10-floxed mice, which has resulted in significant progress toward elucidating the physiologic impacts of ADAM10-mediated cleavage events. By utilizing lck-cre transgenic mice, Tian et al. reported impaired development and suppressed Notch1 signaling in ADAM10-deficient thymocytes(Tian *et al.*, 2008). ADAM10-deficient thymocyte development resembled that of dnKuz and lck-directed Notch1-deficient mice. Additionally, Tian et al. demonstrated that production of the NICD was not detectable in ADAM10-deficient thymocytes. However, in contrast to the previous reports, TCR β expression was not altered by ADAM10 deletion. Thus, the authors concluded that ADAM17 may perform a compensatory role in Notch activation during early stages of DN thymocyte development. However, generation of ADAM17 mutant and conditional knockout mice has not resulted in Notch related phenotypes in lymphocytes (Le Gall *et al.*, 2009; Li *et al.*, 2007a). Interestingly, although the effects of dnKuz and ADAM10 deletion indicate an important Notch-mediated role for ADAM10 in thymocyte development, neither demonstrated altered absolute numbers of T cells in the periphery. Moreover, authors of both reports acknowledged that the modest ADAM10-mediated alterations of thymocyte development do not approach the complete loss of T cell development in mice lacking Notch1 expression in CLPs. They hypothesize that this is dependent on the promoter driving dnKuz or cre expression. Lck-cre did not completely prevent ADAM10 expression in early DN thymocytes. Thus, examining the role of ADAM10 in CLP-commitment to the T cell lineage and the development of early thymocyte precursors must await the generation of other conditional knockout or transgenic mice.

Notch signaling has also been implicated in multiple T cell-mediated autoimmune diseases, including autoimmune lymphoproliferative syndrome (ALPS) and systemic lupus erythematosus (SLE)(Teachey *et al.*, 2008). Additionally, very recent studies have demonstrated a role for Notch ligands, including Dll4, in regulating the differentiation of Th2 and Th17 cells(Mukherjee *et al.*, 2009; Schaller *et al.*, 2007). However, the role of ADAM10 in mature T cell differentiation and activation has not been examined, and may require the use of conditional knockout mice with unaltered thymocyte development.

2.3 B cell development is 'marginal'

B2 lymphocytes, which comprise the majority of circulating B cells, develop from CLPs and differentiate into pro-, then pre-, and ultimately immature B cells prior to exiting the bone marrow (Figure 2). Just as Notch signaling promotes CLP commitment to the T cell lineage, it prevents B lineage fate. Thus, multiple studies have demonstrated that enforced expression of active NICD in bone marrow progenitors completely abrogates B2 cell development (Kawamata *et al.*, 2002; Pui *et al.*, 1999). Following exit from the bone marrow, B2 cells enter a transitional stage and undergo further maturation in the spleen, where the majority differentiate into follicular B cells, while a subset develop into cells of the marginal zone B (MZB) cell lineage, including pre-MZBs and MZB cells. In contrast to T cells, which express Notch1 during development, B lymphocytes preferentially express Notch2, and express minimal levels of Notch1, 3, and 4 (Moriyama *et al.*, 2008; Saito *et al.*, 2003; Santos *et al.*, 2007). By generating B-cell specific Notch2 knockout mice, Saito *et al.* demonstrated that Notch2 signaling is required for development of the MZB cell lineage (Saito *et al.*, 2003), which initiates immune responses to blood-borne infections and transports antigen into the spleen follicles (Pillai *et al.*, 2005). By deleting ADAM10 in a mature B cell-specific manner with CD19-cre knockin mice, Gibb *et al.* also demonstrated an absolute requirement for ADAM10 in MZB development (Gibb *et al.*, 2010). Analysis of Notch target gene expression revealed a dramatic defect in Notch2 signaling. Furthermore, in contrast to wildtype B cells, ADAM10-null cells were completely unresponsive to Dll1-induced Notch stimulation. These findings not only revealed the importance of ADAM10 in B cell development, but also demonstrated that ADAM10 is also responsible for activating RIP-mediated signaling through Notch2. Although S2 cleavage of Notch3 and Notch4 has not been examined, this result suggests that ADAM10 may contribute to their activation as well. In contrast to reports of ADAM10 deletion in thymocytes, deletion in mature B cells did not result in any compensatory Notch2 cleavage by other proteases, including ADAM17. Thus, ADAM10 may be the only protease that can recognize the Notch2 cleavage site, which is distinct from the Notch1 site (Brou *et al.*, 2000).

Interestingly, enhanced Notch2 signaling is a defining characteristic of B cell chronic lymphocytic leukemia (B-CLL), diffuse large B cell lymphoma, and marginal zone lymphoma (Lee *et al.*, 2009; Rosati *et al.*, 2009; Troen *et al.*, 2008). Thus, ADAM10 is a potential target for the treatment of B cell proliferative disease. However the role of ADAM10 in early B cell development in the bone marrow has not been explored, and warrants further study.

2.4 B cell activation, germinal center formation, and antibody production

Recent studies have addressed the role of Notch signaling in mature B cell activation and function. Thomas *et al.* demonstrated that Notch signaling synergizes with B-cell receptor (BCR) and CD40 signaling to enhance murine B cell activation. Upon BCR and CD40 engagement, follicular B cells cultured on OP9 stromal cells expressing Dll1 showed enhanced proliferation and generation of IgG1⁺ cells, when compared to cells cultured on control stroma. Furthermore, *in vivo* experiments showed that Notch signaling contributed to the production of IgG⁺ cells during a T-dependent immune response. Moreover, Dll1-mediated B cell activation was determined to be dependent upon RBP-Jk transcriptional regulation. Follicular B cells isolated from mice expressing a dominant negative form of MAML1, a positive regulator of RBP-Jk, failed to show enhanced proliferation and production of IgG1⁺ cells upon stimulation with anti-CD40 or anti-IgM in the presence of Dll1 (Thomas *et al.*, 2007).

A later study by Santos *et al.* showed that Notch signaling contributes to B cell differentiation to antibody secreting cells (ASC) and also, under some conditions, promotes

class switching. Experiments showed that Dll1-expressing cells enhanced LPS-induced ASC differentiation. Disruption of Notch1 or RBP-Jk reduced ASC recovery, thus, demonstrating that these results are dependent on Notch signaling. Interestingly, the authors also demonstrated that when MZB and B1 cells are cultured in the presence of Dll1, a significant percentage of the cells become ASCs. The authors concluded that Notch signaling by Dll1 could release an inhibitory signal that otherwise maintains B cells in a non-secreting state. This paper also addressed the differential role of Dll1 and Jagged1 (Jg1). Immunohistochemical analysis of murine spleens revealed that Dll1 and Jg1 are both expressed in the marginal zone. However, Dll1 is also expressed in the FDC area within primary and secondary follicles. Results of *ex vivo* cultures suggest that Dll1 enhances Ig secretion, while Jg1 has an inhibitory role (Santos *et al.*, 2007).

In addition to B-cell activation and differentiation to ASCs, Notch signaling is also involved in germinal center formation. Yoon *et al.* recently demonstrated that Notch signaling protects germinal center (GC) B cells from apoptosis. Co-culturing GC B cells with FDC-like cells, HK, which express Dll1 and Jg1, enhanced GC B cell survival. Conversely, blockade of Notch signaling with GSIs increased GC B cell apoptosis. More studies are needed to determine the role of Notch signaling in GC formation *in vivo*. Furthermore, it has yet to be determined whether Notch1 or Notch2 is responsible for promoting GC B cell survival (Yoon *et al.*, 2009).

Although these studies do not address the role of ADAM10, they demonstrate the importance of Notch signaling in regulating B cell survival, activation and differentiation into ASCs. Given that ADAM10 is a critical regulator of Notch2 signaling in B cells, they have generated intriguing questions about the role of ADAM10 in mature B cell differentiation and function.

2.5 Myeloid Development

In the classic model of hematopoiesis, hematopoietic stem cells (HSCs) differentiate to yield common myeloid progenitors (CMPs) or CLPs (Kawamoto and Katsura, 2009). CMPs undergo further differentiation into more mature myeloid cells, including the recently identified myeloid-derived suppressor cells (MDSCs). Because MDSCs suppress anti-tumor responses, myeloid differentiation has been the subject of many recent investigations (Gabrilovich and Nagaraj, 2009). To date, numerous hematopoietic pathways, including Notch signaling, have been implicated but remain controversial. Several investigators have reported that alterations in Notch signaling have minimal effects on the myeloid compartment (Carlesso *et al.*, 1999; Pui *et al.*, 1999; Stier *et al.*, 2002). Yet, Kawamata *et al.* reported that constitutive Notch signaling promotes myeloid differentiation in a non-cell autonomous manner (Kawamata *et al.*, 2002). This is supported by a report of abrogated B cell and myeloid cell development in mice deficient in downstream targets of Notch (Schroeder *et al.*, 2003). However, other studies have indicated that Notch signaling inhibits myeloid differentiation (Bigas *et al.*, 1998; Qyang *et al.*, 2004). Qyang *et al.* demonstrated that blockade of Notch signaling at the γ -secretase cleavage site induces myeloid accumulation (Qyang *et al.*, 2004).

Many of these alterations were observed in mice with altered lymphocyte development. This suggests that Notch signaling may modulate the differentiation pathway of CLPs and CMPs from a common upstream progenitor. Two groups have recently described common myelo-lymphoid progenitors (CMLPs) that are indistinguishable from HSCs (Chi *et al.*, 2009; Kawamoto and Katsura, 2009). Although these multipotent cells often commit to the B or T cell lineage, they retain the potential for myeloid development (Kawamoto and Katsura, 2009). Therefore, alterations in Notch signaling during lymphocyte development could also affect myelopoiesis. Collectively, these findings indicate that myeloid differentiation may be

regulated by the signal strength and temporal stage of Notch signaling. Therefore, examination of other Notch regulators, including ADAM10, could clarify the role of Notch signaling in myeloid differentiation.

3. ADAM10 shedding and autoimmunity

Although the role of ADAM10 in autoimmune disease has not been directly examined to date, numerous putative ADAM10-substrates are known contributors to autoimmune reactions and inflammation.

3.1 CD23

The low affinity IgE receptor, FcεRII or CD23, is a type II transmembrane Fc receptor expressed by B cells, follicular dendritic cells (FDCs) and some human myeloid cells. Numerous studies have implicated CD23 as a natural regulator of IgE production and IgE-mediated Type I hypersensitivity reactions, including allergic airway inflammation. Binding of IgE to CD23 on B cells is thought to transduce an inhibitory signal that suppresses further IgE synthesis (Figure 3). However, ADAM10 cleavage of CD23 may interrupt this negative feedback mechanism, resulting in elevated IgE levels (Conrad *et al.*, 2007). In fact, treatment of immunized mice with the metalloprotease inhibitor, batimistat, not only inhibited CD23 cleavage, but also markedly reduced IgE production (Christie *et al.*, 1997). We have recently shown that this cleavage occurs primarily intracellularly and both the CD23 and the ADAM10 are incorporated into exosomes (Mathews *et al.*, 2010).

Additionally, the soluble cleaved product, sCD23, is a contributing mediator in rheumatoid arthritis. CD23^{-/-} mice have a delayed onset and reduced severity of collagen-induced arthritis (CIA), and treatment of wildtype mice with anti-CD23 antibodies markedly attenuates disease signs (Kleinau *et al.*, 1999; Plater-Zyberk and Bonnefoy, 1995). Moreover, sCD23 is elevated in synovial fluids of patients with rheumatoid arthritis, and is associated with erosive status (Ribbens *et al.*, 2000). sCD23 may contribute to rheumatic disease by activating monocytes via interactions with CD11c-CD18 (Lecoanet-Henchoz *et al.*, 1995). Because of these findings, inhibiting cleavage by the endogenous CD23 sheddase has been proposed as a novel therapy for controlling allergic and rheumatic disease (Conrad *et al.*, 2007).

CD23 monomers, consisting of a short cytoplasmic tail, a transmembrane portion, an extracellular stalk region forming a coiled-coil structure, and a lectin head domain that binds IgE, form a homo-trimeric tertiary structure, which is stabilized by IgE binding. This stabilization can be mimicked by antibody binding to the lectin heads. Anti-lectin antibodies have been shown to inhibit CD23 cleavage and airway inflammation in a mouse asthma model (Dasic *et al.*, 1999; Munoz *et al.*, 1998). However, in the absence of ligand, monomers are highly susceptible to proteolytic cleavage within the stalk domain. Demonstration that CD23 cleavage is sensitive to hydroxamic acid inhibitors, which inhibit the proteolytic activity of ADAMs, stimulated significant progress toward identifying the sheddase (Conrad *et al.*, 2007). Although early studies implicated the proteolytic activity of ADAMs 8 and 15 (Fourie *et al.*, 2003), subsequent studies ruled out a role for ADAMs 8, 9, 12, 15, and 17 *in vivo* (Le Gall *et al.*, 2009; Weskamp *et al.*, 2006). Two independent groups utilized ADAM10-null MEFs and pharmacologic inhibitors to demonstrate that ADAM10 activity is responsible for CD23 cleavage (Lemieux *et al.*, 2007; Weskamp *et al.*, 2006). However, (Jackson *et al.*, 2009) later reported that MMP-9 is the principal sheddase of CD23 in LPS-treated mice, calling into question the physiologic relevance of ADAM10-mediated CD23 cleavage. Thus, Gibb et al. examined CD23 cleavage in B cell-specific ADAM10-deficient mice. Flow cytometric and immunohistochemical analysis revealed a remarkable increase in CD23 levels expressed on ADAM10-null B cells, while ELISAs illustrated a significant

decrease in serum sCD23. Additionally, destabilization of CD23 trimers with anti-stalk CD23 antibodies only stimulated cleavage from control B cells without influencing cleavage from ADAM10-deficient cells (Gibb *et al.*, 2010). The seemingly contradictory reports on MMP-9 and ADAM10-mediated cleavage may be explained by the use of LPS-treated mice by Jackson *et al.* (Jackson *et al.*, 2009). Although analysis of ADAM10-deficient B cells demonstrated a clear role for ADAM10 in constitutive cleavage of CD23 from naïve B cells, LPS-induced activation may allow ADAM10-independent cleavage.

The impact of ADAM10-mediated CD23 cleavage on allergic and rheumatic disease remains the focus of current studies. Because ADAM10 has multiple substrates, including Notch, the response of ADAM10-null B cells in disease states might not be attributed to CD23. Thus, the generation of novel *in vivo* models is required to further elucidate the role of ADAM10-mediated cleavage events in allergic and rheumatic inflammation.

3.2 CD44

CD44 constitutes numerous cell adhesion proteins that are generated by alternative splicing of CD44 mRNA. Many CD44 transmembrane proteins, expressed by most inflammatory leukocytes, bind hyaluronan (HA) in the extracellular matrix. Inflammation-induced upregulation of CD44 by endothelial cells can cause leukocyte adhesion to inflamed tissue via CD44-HA-CD44 bridged binding. Cleavage of CD44 is thought to allow detachment of leukocytes, including T cells, from the endothelium and transmigration into inflamed tissue. Anti-CD44 antibodies have reduced the severity of numerous models of autoimmunity in mice, including CIA, type 1 diabetes in NOD mice, and experimental autoimmune encephalomyelitis (EAE)(Naor *et al.*, 2007). Conversely, levels of the cleaved CD44 ectodomain are elevated in patients with rheumatoid arthritis. Interestingly, CD44 cleavage not only alters leukocyte adhesion and migration by reducing CD44 surface levels, but also activates RIP-mediated CD44 signaling. The intracellular domain of CD44 can alter gene expression and may result in various cell processes that cause leukocyte activation, proliferation, or apoptosis. Cleavage of CD44 also regulates migration and invasion by tumor cells, and was recently reviewed by Stamenkovic and Yu(Stamenkovic and Yu, 2009). Multiple MMPs, ADAM10, and ADAM17 have all been shown to cleave CD44 in multiple cell types. However, a recent report demonstrated that only ADAM10 can cleave CD44 in melanoma cells(Anderegge *et al.*, 2009). Authors of an additional study utilizing ovarian carcinoma cells concluded that ADAM17 was responsible for CD44 release from the cell surface, while ADAM10 processed CD44 within the endosomal pathway(Stoeck *et al.*, 2006). Additional studies of CD44 cleavage revealed a novel function of ADAM10, discussed below. However, the physiologic consequences of ADAM10-mediated CD44 processing have not been examined *in vivo*.

3.3 Chemokine shedding

Catabolic shedding of membrane-bound chemokines is an important processing event that regulates inflammatory responses. Results of several studies indicate that ADAM10 is responsible for release of fraktalkine (CX3CL1) and CXCL16 from the cell surface. The transmembrane form of fraktalkine facilitates leukocyte adhesion to endothelial cells by interacting with its receptor CX3CR1, while fraktalkine cleavage allows detachment(Hundhausen *et al.*, 2007). Additionally, soluble fraktalkine is a chemoattractant for T cells and monocytes(Bazan *et al.*, 1997). Elevated levels have been reported in multiple autoimmune states, including autoimmune exocrinopathy, neuropsychiatric lupus, and EAE(Huang *et al.*, 2006; Sato *et al.*, 2006; Tsubota *et al.*, 2009). Additionally, fraktalkine inhibition ameliorates experimental autoimmune myositis and CIA in mice(Nanki *et al.*, 2004; Suzuki *et al.*, 2005).

CXCL16 is thought to regulate atherogenic inflammation, because the membrane-bound form can function as a scavenger for oxidized low density lipoprotein (oxLDL)(Sawamura *et al.*, 1997), and the soluble form can attract NKT cells to inflamed tissue(Johnston *et al.*, 2003). Elevation of soluble CXCL16 is considered a marker for atherosclerosis and coronary disease(Lehrke *et al.*, 2007), while inhibition of CXCL16 can suppress antibody-mediated glomerulonephritis (Garcia *et al.*, 2007). Thus, CXCL16 cleavage could promote arterial inflammation that underlies multiple disease states.

In vitro studies have demonstrated that siRNA or pharmacologic inhibition of ADAM10 can significantly impair cellular release of CXCL16(Abel *et al.*, 2004; Gough *et al.*, 2004). Accordingly, INF- γ and TNF- α induce soluble CXCL16 production from wildtype MEFs but do not influence release by ADAM10-deficient cells(Abel *et al.*, 2004). Two studies extended ADAM10's role in chemokine release to fraktalkine by demonstrating diminished fraktalkine release from ADAM10-null MEFs and transfected COS-7 cells treated with an ADAM10 inhibitor(Ludwig *et al.*, 2005; Schulte *et al.*, 2007a). Schulte et al. also described sequential processing of both chemokines by ADAM10 and γ -secretase, which indicates an additional role for chemokine intracellular domains(Schulte *et al.*, 2007a). Although these results indicate that ADAM10 performs an important role in chemokine processing and subsequent immune responses, this has not been examined *in vivo*. Thus, elucidating the physiologic consequences of ADAM10-mediated chemokine release awaits analysis of experimental autoimmunity in conditional knockout or transgenic mice.

3.4 FasL and TNF- α

Binding of the Fas receptor, a ubiquitously expressed member of the TNF receptor family, with Fas ligand (FasL) expressed on adjacent activated T cells and NK cells triggers apoptosis(Strasser *et al.*, 2009). Mice lacking Fas or FasL develop lymphadenopathy and SLE-like autoimmune disease(Takahashi *et al.*, 1994; Watanabe-Fukunaga *et al.*, 1992). Additionally, a subset of ALPS patients have an inherited mutation in the Fas gene(Fisher *et al.*, 1995; Rieux-Laucat *et al.*, 1995). Thus, FasL:Fas interactions induce cell death of infected target cells, maintain lymphocyte homeostasis, and regulate lymphocyte expansion by mediating activation-induced cell death (AICD). Cleavage of FasL, a member of the TNF cytokine family, is thought to be an important mechanism regulating apoptosis, since soluble FasL is elevated in serum of patients with dysregulated inflammatory diseases. Although multiple proteases have been implicated in FasL cleavage, Schulte et al. initially demonstrated that ADAM10 inhibition significantly reduces FasL shedding from primary human T cells(Schulte *et al.*, 2007b). The subsequent increase in FasL cell surface expression increased the efficiency of T cell-mediated killing of target cells and AICD. Additionally, Kirkin et al. described ADAM10-mediated RIP of FasL, leading to release of an intracellular domain that inhibits gene transcription(Kirkin *et al.*, 2007). These results indicate that ADAM10-mediated FasL cleavage downregulates Fas-mediated apoptosis. However, other proteases have been implicated in FasL cleavage(Mitsiades *et al.*, 2001; Powell *et al.*, 1999). Thus, elucidating the biologic relevance of ADAM10-mediated FasL processing awaits *in vivo* examination of ADAM10-deficient activated T cells.

TNF- α is also released from cell surfaces following cleavage of pro-TNF- α . Two early reports described a role for ADAM10 in this process(Lunn *et al.*, 1997; Rosendahl *et al.*, 1997). Additionally, Arduise et al. recently reported that ADAM10 is responsible for TNF- α release in response to antibody binding to tetraspanins(Arduise *et al.*, 2008). However, the vast majority of data demonstrates that most TNF- α secretion is dependent upon ADAM17 activity *in vitro* and *in vivo*(Black *et al.*, 1997).

3.5 Lag-3

In early stages of T cell activation, effector molecules are upregulated, while inhibitory proteins are inactivated. Lymphocyte activation gene-3 (Lag-3) is an inhibitory transmembrane protein expressed by activated T cells and NK cells that binds MHC class II molecules with a higher affinity than CD4. It regulates the suppressive activity of regulatory T cells and controls activation-induced effector T cell expansion (Huang *et al.*, 2004; Workman *et al.*, 2004). Following T cell activation *in vivo*, soluble Lag-3 accumulates in mouse serum. Li *et al.* reported that Lag-3 cleavage is required for antigen-specific T cell activation, as non-cleavable mutants prevent proliferation and cytokine production (Li *et al.*, 2007b). The authors also demonstrate that cleavage is mediated by ADAM10 and ADAM17. ADAM10 is responsible for constitutive and activation-induced cleavage, while ADAM17 mediates PKC θ -dependent cleavage. Additionally, ADAM10 siRNA suppressed T cell proliferation in a Lag-3 dependent manner. One report also described Lag-3 expression on activated B cells (Kisielow *et al.*, 2005). Thus, Lag-3 cleavage may also regulate B cell proliferation. Li *et al.* (Li *et al.*, 2007b) have provided the most direct evidence describing a role for ADAM10 activity in T cell activation, and have raised intriguing questions about ADAM10's role in adaptive immune responses.

4. Regulation of ADAM10 activity

Endogenous regulation of ADAM10 activity has not been thoroughly examined. ADAM10 is expressed as a zymogen. Cleavage of the pro-domain by a furin protease results in ADAM10 activation (Hwang *et al.*, 2006). Following pro-domain removal, regulation of ADAM10-mediated cleavage is dependent on the structural conformation of the substrate. If the cleavage site is exposed, cleavage is thought to occur constitutively. However, several reports have indicated that proteolytic activity is enhanced by retinoic acid receptor signaling, PKC signaling, nardilysin expression, cholesterol depletion, N-glycosylation, and calcium influx (Escrivente *et al.*, 2008; Hiraoka *et al.*, 2007; Kojro *et al.*, 2001; Sanderson *et al.*, 2005; Skovronsky *et al.*, 2000; Tippmann *et al.*, 2009). Thus, ionomycin is routinely used to enhance ADAM10-mediated cleavage (Le Gall *et al.*, 2009). Additionally, inhibition of proteolytic activity by tissue inhibitors of metalloproteinases 1 (TIMP1), TIMP3, and the reversion-inducing cysteine-rich protein with Kazal motifs (RECK) has been reported (Amour *et al.*, 2000; Muraguchi *et al.*, 2007). These regulatory mechanisms may represent opportunities for pharmacologic intervention of autoimmune disease states in which ADAM10 substrates contribute to pathogenesis or disease progression.

5. Novel functions of ADAM10

In addition to ectodomain shedding and RIP, two novel functions of ADAM10 were recently described. Examination of cellular release of CD44 and another ADAM10 substrate, L1, has revealed that ADAM10 contributes to exosomal sorting of substrates. Stoeck *et al.* demonstrated that L1 and CD44, thought to be cleaved from the cell membrane, were actually cleaved in endosomal vesicles that were subsequently released in the form of exosomes (Stoeck *et al.*, 2006). Additionally, Sharples *et al.* described the release of another ADAM10 substrate, APP, in exosomes (Sharples *et al.*, 2008), and both reports also demonstrated that ADAM10 itself is sorted into exosomes. Although exosomal transport of many substrates has not been examined, it is possible that ADAM10 is also responsible for the inclusion of other substrates in exosomes. Although the exact role of exosomes in immune reactions may depend on exosomal content, many exosomal proteins maintain their biological function. Exosome release has also been shown to increase upon lymphocyte and dendritic cell activation (Thery *et al.*, 2002). Thus, exosomes may have a role in the enhancement of autoimmune reactions. Conversely, multiple studies have described the ability of dendritic cell-derived exosomes to attenuate the severity of autoimmune disease

models in mice (Bianco *et al.*, 2007). Nevertheless, this novel role of ADAM10 in exosomal sorting may modulate autoimmune reactions.

Finally, Tousseyn *et al.* recently demonstrated that while ADAM10 mediates RIP of numerous other substrates, ADAM10 itself is also susceptible to RIP by ADAMs 9 and 15 and γ -secretase (Tousseyn *et al.*, 2009). The intracellular domain of ADAM10 was observed in the nucleus, localized to nuclear speckles thought to represent sites of gene regulation. Although the biological consequences of this potential signaling cascade have not been examined, this finding illustrates that alterations in ADAM10 expression may have effects independent of ADAM10 substrates. Moreover, it suggests that blockade of ADAM10 activity and ADAM10 expression may have differential effects.

6. Conclusion

Through the development of hydroxamate inhibitors, dominant negative constructs, and protease-deficient MEFs, a multitude of putative ADAM10 substrates with known roles in inflammation and autoimmunity have been identified. The generation of conditional knockout mice has revealed the physiologic relevance of several ADAM10-mediated cleavage events, but most await further analysis. Initiation of Notch-dependent development has been the most striking observation in lymphocyte studies. Although interesting, these developmental phenotypes may present challenges for the examination of ADAM10's role in adaptive immunity and autoimmunity. However, the generation of novel *in vivo* models should overcome this hurdle and allow further examination of ADAM10 as a potential target for the treatment of lymphoproliferative and autoimmune diseases.

Abbreviations

DII	delta-like
NICD	Notch intracellular domain
RIP	regulated intramembrane proteolysis
NRR	negative regulatory region
CMP	common myeloid progenitor
HSC	hematopoietic stem cell
CLP	common lymphoid progenitor

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Figure 1. ADAM10-mediated RIP of Notch2 is required for Marginal Zone B cell development

Signaling through the Notch2 receptor expressed on transitional B cells directs marginal zone B cell differentiation (Saito *et al.*, 2003). The Notch2 heterodimer on B cells binds ligands, Jagged 1-2 and Delta-like 1, present on stromal and antigen presenting cells. Binding initiates sequential cleavage events by ADAM10 (S2 cleavage) and a γ -secretase complex (S3 cleavage). Cleavage releases the Notch2 intracellular domain (N2ICD), containing RAM (yellow), nuclear localization (red), transactivating (TAD) (blue), EGF-repeat (black), and PEST domains (green). Transport of the N2ICD to the nucleus followed by binding to the transcription factor, RBP-J κ , allows the release of co-repressors (CoR), and attraction of co-activators (CoA) to the transcriptional complex. The activated complex transcribes Notch target genes, including CD21/35, Deltex-1, Hes 1, and Hes 5, that promote development of marginal zone B cells. Deletion of ADAM10, Notch2, and RBP-J κ from B cells, or Delta-like 1 from stromal cells prevents marginal zone B cell development.

Figure 2. ADAM10 and Notch similarly regulate B cell development

Precursors to the B cell lineage develop from common lymphoid progenitors (CLPs) in the bone marrow. Constitutive expression of the Notch intracellular domain (NICD), Hes 1 or Hes 5 in CLPs prevents B lineage commitment, while promoting T cell development. Development of the marginal zone B cell (MZB) lineage is regulated by Notch2 signaling. The majority of transitional cells differentiate into follicular B cells, which constitute the majority of circulating B cells. However, ADAM10-mediated Notch2 signaling promotes MZB lineage development. HSC-hematopoietic stem cell, CMP-common myeloid progenitor, Im-Immature. Solid arrows and dashed arrows indicate confirmed and hypothetical differentiation events, respectively.

Figure 3. Ectodomain shedding of CD23, a regulator of IgE-mediated inflammation
 CD23 monomers, consisting of a short cytoplasmic tail, a transmembrane portion, an extracellular stalk region forming a coiled-coil structure, and a lectin head domain that binds IgE, form a homo-trimeric tertiary structure, which is stabilized by IgE binding. In the absence of IgE or the presence of anti-stalk antibodies, ADAM10 constitutively cleaves CD23 ectodomains and releases soluble CD23 (sCD23). Cleavage prevents IgE:CD23 interaction at the B cell surface, and results in elevated IgE synthesis. This figure was modified from Conrad et al.(Conrad *et al.*, 2007).