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ADAM10 Overexpression Shifts Lympho- and Myelopoiesis by Dysregulating Site 2/Site 3 Cleavage Products of Notch

David R. Gibb,*1 Sheinei J. Saleem,*1 Dae-Joong Kang,* Mark A. Subler,† and Daniel H. Conrad*

Although the physiological consequences of Notch signaling in hematopoiesis have been extensively studied, the differential effects of individual notch cleavage products remain to be elucidated. Given that ADAM10 is a critical regulator of Notch and that its deletion is embryonically lethal, we generated mice that overexpress ADAM10 (ADAM10 transgenic [A10Tg]) at early stages of lympho- and myeloid development. Transgene expression resulted in abrogated B cell development, delayed T cell development in the thymus, and unexpected systemic expansion of CD11b+Gr-1+ cells, also known as myeloid-derived suppressor cells. Mixed bone marrow reconstitution assays demonstrated that transgene expression altered hematopoiesis via a cell-intrinsic mechanism. Consistent with previously reported observations, we hypothesized that ADAM10 overexpression dysregulated Notch by uncoupling the highly regulated proteolysis of Notch receptors. This was confirmed using an in vitro model of hematopoiesis via culturing A10Tg hematopoietic Lineage Sca-1+c-Kit+ cells with OP-9 stromal cells in the presence or absence of Delta-like 1, a primary ligand for Notch. Blockade of the site 2 (S2) and site 3 (S3) cleavage of the Notch receptor demonstrated differential effects on hematopoiesis. OP9-DL1 cultures containing the ADAM10 inhibitor (S2 cleavage site) enhanced and rescued B cell development from wild-type and A10Tg Lineage Sca-1+c-Kit+ cells, respectively. In contrast, blockade of γ-secretase at the S3 cleavage site induced accumulation of the S2 product and consequently prevented B cell development and resulted in myeloid cell accumulation. Collectively, these findings indicate that the differential cleavage of Notch into S2 and S3 products regulated by ADAM10 is critical to hematopoietic cell-fate determination.

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D isintegrin and metalloproteinases (ADAMs) regulate cell signaling pathways by cleaving the extracellular domains of membrane-bound receptors and ligands. Consequently, these proteins serve as initiators for signaling pathways that require regulated intramembrane proteolysis (RIP) of receptor:ligand complexes. In vitro-based assays have revealed that ADAM10 is an important mediator of ectodomain shedding and RIP of numerous substrates, such as the low-affinity IgE receptor CD23 and the Notch ligand Delta-like 1 (DL1). Proteolytic processing of these substrates contributes to the pathogenesis of multiple disease states, including allergy, cancer, and inflammation (1, 2). Accordingly, there is growing interest in ADAM10 as a pharmacological target for these conditions. However, determination of the physiologic consequences of ADAM10-mediated cleavage events has been limited by lethality of ADAM10-null murine embryos (3).

Production of ADAM10-deficient embryos and conditional knockout mice has demonstrated a critical role for ADAM10 in developmental pathways, including thymocyte and marginal zone B cell development (4, 5). Each report concluded that impaired development in the absence of ADAM10 was the result of diminished Notch signaling, which depends on RIP for signal activation.

Many studies have demonstrated the importance of Notch signaling in lymphocyte development. The Notch signaling pathway is highly conserved, consisting of four families of receptors (Notch1–4) that interact with ligands (Jagged and Delta) expressed by neighboring cells (6–8). Following ribosomal synthesis, the Notch receptor undergoes a furin-mediated maturation at site 1 in the Golgi apparatus prior to trafficking to the cell surface. At the surface, Notch is expressed as an integral membrane protein, consisting of both extracellular (NEXT) and intracellular domains (NICD). Once engaged with its ligand, the extracellular domain undergoes an ADAM10-mediated cleavage at site 2 (S2). This event generates a substrate for γ-secretase complex to perform a final cleavage of Notch at site 3 (S3), releasing the transcriptionally active NICD (Fig. 6) (9). Several studies have reported the accumulation of intact receptor and the S2 product as a result of ADAM10 and γ-secretase blockade, respectively (10, 11). Although inhibition of both pathways prevents NICD activation, the consequences of accumulation of these different cleaved products on hematopoiesis remains to be determined.

Notch is critical for T cell lineage commitment and maturation as well as marginal zone B cell development (6, 12). Notch1 signaling
in common lymphoid progenitors (CLPs) is required for early thymocyte development (13), and enforced signaling enhances T cell development (12). In humans, mutations in the negative regulatory region of the Notch1 receptor allow ligand-independent cleavage, resulting in excessive Notch1 signaling and the formation of T cell acute lymphocytic leukemia (T-ALL) (9). Several groups report the use of γ-secretase inhibitors (GSIs) as a means of suppressing the expansion of T-ALL cells and thus as a potential method for treating T-ALL and other Notch-dependent lymphoproliferative disorders (14, 15). However, GSI treatment does not completely abrogate Notch signaling, because it does not affect the initiation of the signaling pathway mediated by ADAMs. GSI treatment results in the accumulation of the S2 cleavage product, and its pathologic consequences remain to be elucidated (8, 16).

In contrast to T cell development, Notch signaling prevents commitment of CLPs to the B cell lineage (12). Furthermore, enforced expression of constitutively active NICD or Notch target genes in BM progenitors abrogates B lineage commitment and promotes noncell autonomous expansion of CD11b+Gr-1+ myeloid cells in transplant recipients (17, 18).

Although the effect of Notch signaling on lymphopoiesis has been well established, its role in myelopoiesis remains controversial. Several studies have shown that Notch signaling promotes expansion of undifferentiated myeloid cells (19), abrogates B cell development, and promotes immature myeloid cell formation (17). In addition, mice deficient in downstream Notch effectors exhibit defective B cell and myeloid development (20). Collectively, these observations suggest that Notch-mediated alterations in lymphocyte development could serve to modulate myelopoiesis. This supposition is supported by recent data demonstrating that myeloid potential is retained in developing lymphocytes (21, 22). Nonetheless, other studies either have failed to detect myeloid alterations under the same conditions as the aforementioned studies (23) or have indicated that Notch1 and Notch2 are capable of inhibiting myeloid differentiation (24). Adding to this ambiguity is the observation that mice with diminished γ-secretase activity exhibit splenomegaly as a result of the accumulation of myeloid cells (25). The presence of multiple conflicting data suggests that myeloid differentiation may depend on both the strength and the temporal stage at which Notch signaling occurs.

Many disease states are characterized by the excessive production of ADAM10 cleavage products (2). Inspired by the strength and the temporal stage at which Notch signaling occurs, recipient mice were generated as described in the supplemental methods. A10Tg mice were generated as described in the supplemental methods. ADAM10 overexpression was found to alter hematopoiesis by dysregulating RIP-dependent Notch signaling. Taken together, these observations underscore the importance of ADAM10 in Notch-mediated signaling for both lympho- and myelopoiesis.

Materials and Methods

**Mice**

A10Tg mice were generated as described in the supplemental methods. C57BL/6 and congenic CD45.1+ (B6-Ly5.2) mice were purchased from the National Cancer Institute. All mouse protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

**Flow cytometry and cell sorting**

Cell isolation and labeling was conducted as described previously (5). In addition, peritoneal fluid cells were obtained by flushing the peritoneal cavity with PBS (5 ml). BM cells were isolated by flushing excised tissues and femurs with complete RPMI 1640 medium. Single-cell suspensions of peripheral lymph node (PLN) cells, thymocytes, and splenocytes were created by disrupting inguinal, brachial, axillary lymph nodes, thymus, and spleens, respectively, with glass slides. Cells were labeled following RBC lysis and filtration through 40 μm cell strainers. Abs included anti-mouse unlabeled 2.4G2, biotinylated CD135 (A2F10), FITC-conjugated IL-7R (A7R34), CD44 (IMT7), B220 (RA3-6B2), and Gr-1 (RB6-8C5); PE-conjugated B220, CD8 (53-6.7), c-Ki-PE (2B8); PE/Cy7-conjugated CD11b (M1/70), and CD3e (2C11); allopurinol-labeled B220, CD4 (RM4-5), CD5 (53-7.3), CD45.2 (104), and εεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεee
control of the H-2Kb promoter and the IgH enhancer region (Supplemental Fig. 1A). These transcriptional regulatory units were previously used to generate multiple transgenic mouse lines, including TCR, CD23, and bkl transgens. Thus, this vector allows expression in early lymphocyte progenitors (28–30). Inclusion of the IgH enhancer results in preferential expression on B lineage cells. Two founder lines, F240 and F258, were generated, and Southern blot analysis of genomic DNA from F2 progeny demonstrated that both lines contain similar copy numbers of the transgene (Supplemental Fig. 1B). Because progeny of both lines have nearly identical phenotypes, the following data are presented from line F240, unless otherwise stated.

**ADAM10 overexpression prevents B2 lymphocyte development and inhibits development of thymocyte progenitors.**

Western blot and flow cytometric analysis demonstrated that transgene expression resulted in elevated levels of ADAM10 in BM cells, including pro-/pre-B cells (B220⁺IgM⁻) (Supplemental Fig. 1C–E). Unexpectedly, overexpression markedly reduced the levels of pro-/pre-B cells and immature B cells (B220⁺IgM⁺) in BM (Supplemental Fig. 1C). This led to a near complete loss of peripheral B cells in peripheral organs including blood, lymph nodes, and spleen (Fig. 1A; data not shown). Analysis of B cells from peritoneal fluid revealed that levels of B1a (B220⁺CD11b⁺CD5⁺) and B1b cells (B220⁺CD11b⁺CD5⁻) in A10Tg mice were not significantly altered compared with littermate (LM) controls, whereas B2 cells (B220⁺CD11b⁺CD5⁻) were nearly absent (Fig. 1B). Thus, the block in B cell development was specific to BM-derived B2 cells. In addition, transgene expression suppressed development of thymocyte progenitors. A 10-fold reduction was observed in Lin⁺CD44⁺c-Kit⁺ early thymocyte progenitors (ETPs) in A10Tg BM compared with LM (Fig. 1C), which resulted in reduced levels of A10Tg thymic ETPs (data not shown) (31, 32). Accordingly, A10Tg mice have reduced levels of total, double-negative, and double-positive thymocytes and a small thymus. However, numbers of single-positive thymocytes and peripheral T cells in the PLN and the spleen were not altered in A10Tg mice (Fig. 1D).

**MDSC accumulation in A10Tg mice**

The blockade of B2 cell development was expected to result in reduced levels of total splenocytes. However, the spleens of A10Tg mice were noticeably enlarged, weighing an average of 2.5-fold more than LM spleens (Fig. 2A), containing twice as many nucleated cells (data not shown). The forward and side scatter pattern of A10Tg splenocytes indicated the presence of large granular myeloid cells (Fig. 2B). Further analysis, as shown in Fig. 2C, revealed that ~63% of transgenic splenocytes were CD11b⁺Gr-1⁺ cells, compared with 53.5% of LM splenocytes. Although the majority of WT CD11b⁺Gr-1⁺ cells differentiate into mature myeloid cells prior to exiting the BM, A10Tg CD11b⁺Gr-1⁺ cells expanded in BM, constituting 93.5% of BM cells, and entered the spleen and the PBL at dramatically high levels (Fig. 2C). CD11b⁺Gr-1⁺ cells outside the bone marrow (BM) are classified as MDSCs, consisting of monocytic (CD11b⁺Gr-1⁺Ly6G⁻) and granulocytic subsets (CD11b⁺Gr-1⁺Ly6G⁺) (33). Light micrographs of sorted A10Tg CD11b⁺Gr-1⁺ splenocytes and flow cytometry confirmed that A10Tg mice contain both monocytic and granulocytic MDSCs in the PBL and spleen (Fig. 2D–F).

**Hematopoietic expression of ADAM10 alters development via a cell-intrinsic mechanism.**

To confirm that the observed phenotype was the result of transgene expression by hematopoietic cells and not a dysregulation in the BM stromal environment, mixed BM assays were conducted. As controls, irradiated CD45.2⁺ A10Tg hosts were reconstituted with

![FIGURE 1. ADAM10 overexpression prevents B2 cell development and suppresses development of thymocyte progenitors. A and B, Flow cytometric analysis of T cells (CD3⁺) and B cells (B220⁺) in spleen (A) and B cell subsets in peritoneal fluid (B). Lower two panels are gated on B220⁺ cells in upper histograms. B cells, B220⁺CD11b⁺CD5⁻; B1a cells, B220⁺CD11b⁺CD5⁺; and B1b cells, B220⁺CD11b⁺CD5⁻. C, 1× representative thymi from indicated mice. BM cells were analyzed for Lin⁻CD25⁻CD44⁺CD4⁻CD8⁻ ETPs. Lineage mixture includes B220, Ter-119, CD11b, Gr-1, CD3, CD4, and CD8. D, Amount of thymocyte subsets and CD3⁺ T cells in the spleen and PLN. n = 4, mean ± SEM, DN: CD4⁻CD8⁻, DP: CD4⁺CD8⁺, CD4⁺CD8⁻, CD8⁻CD4⁺, CD8⁻CD4⁻. Dot plots and histograms are representative of six (A), four (B), and three (C) independent experiments. Numbers on plots indicated percentage of gated cells within box. *p < 0.05.]
LSK cells from WT CD45.1+ congenic mice, and irradiated WT
CD45.1+ congenic hosts were reconstituted with CD45.2+ A10Tg
LSK BM cells. Despite being in a WT host, A10Tg BM re-
capitulated the observed altered hematopoiesis of A10Tg mice. In
addition, WT BM cells demonstrated normal cell differentiation,
even in an A10Tg host (data not shown). This finding indicated
that altered cell differentiation in A10Tg mice was due to alter-
ations in signaling pathway(s) within hematopoietic cells rather
than the microenvironment. To further confirm this, mixed BM
chimeras were performed. Irradiated CD45.1+ WT hosts were
reconstituted with a mixture of LSK BM cells from CD45.2+
A10Tg and CD45.1+ WT donors. This resulted in similar re-
constitution of host BM by A10Tg and WT donor cells 42 and
63 d after cell transfer. However, development from BM into
peripheral lymphoid organs was less efficient in A10Tg cells,
as ~66 ± 5.1% (SE) and 81 ± 6.2% of recipient spleen and
PLN cells, respectively, developed from WT BM at day 42. The
selective development of WT thymocytes was most striking, as
98.1 ± 1.0% of thymocytes at day 63 were of WT origin (data
not shown). This result supports the diminished thymocyte de-
velopment in A10Tg mice (Fig. 1C, 1D). Despite these differ-
ences, hematopoietic development of CD45.1+ WT cells was
similar to development in LM control mice, and development of
CD45.2+ A10Tg cells was closely mimicked development in A10Tg
cells. MDSCs only expanded from A10Tg BM cells, and B lin-
eage cells predominantly differentiated from WT BM (Fig. 3). These
results demonstrate that ADAM10-mediated MDSC ex-
ansion is not the indirect result of abrogated B cell development,
trans-cleavage of BM stromal cell ligands, or cytokine secretion,
which would cause WT MDSC expansion. They also illustrate that
ADAM10 overexpression on hematopoietic cells causes MDSC
expansion via an intrinsic cell autonomous mechanism.

ADAM10 prevents commitment of CLPs to the B cell lineage
Expansion of MDSCs in conjunction with blockade of B2 cell
development indicated that ADAM10 regulates the commitment of
BM progenitors to myeloid or lymphoid lineages. According to the
classical model of hematopoiesis, HSCs, which are LSK cells in
the BM, develop into common myeloid progenitors (CMPs) or
CLPs, giving rise to early thymocyte progenitors or pro-B cells
(34). Supplemental Fig. 1C illustrates that a small percentage of
B220+ cells was present in A10Tg BM. However, further analysis
revealed that the few B220+CD19+ cells in A10Tg mice also
expressed the myeloid markers CD11b and Gr-1 (data not shown),
indicating that alterations in hematopoiesis occur prior to the pro-
B cell stage. Thus, to determine the stage at which ADAM10 over-
expression alters hematopoiesis, levels of LSKs, CLPs, and CMPs
were examined. Analysis of BM lineage-positive cells (Ter119,
CD3ε, CD11b, and Gr-1) and CD19+ cells demonstrated the near
absence of Lin-CD19+ B cells in A10Tg mice. However, the
percentage of Lin-CD19+ cells was similar to LM levels (Fig.
4A). Lin-CD19+ LM cells contain B220+ B cell precursors,
which were absent from A10Tg BM (Fig. 4B). Analysis of IL-7Rα
cells revealed a modest decrease in CLPs (Lin-CD19+ B220+ IL
7Rα-Kit+Scal+ in A10Tg BM (Fig. 4C). In addition, Inlay et al.
(35) recently demonstrated that Ly6D+ CLPs are committed to the
B cell lineage, whereas Ly6D+ CLPs are uncommitted lymphoid
progenitors. Accordingly, 30% of LM CLPs expressed high levels

FIGURE 2. ADAM10 overexpression causes the expansion of MDSCs. A, 1× representative spleens and average spleen weight of indicated mice, n = 4, mean ± SEM. Flow cytometric analysis of forward scatter (FS) versus side scatter (SS) of splenocytes (B), the percentage of CD11b+Gr-1+ MDSCs present in the BM, PBL, and spleen (SPL) (C), and the percentage of CD11b+Ly6-G- and CD11b+Ly6-G+ MDSCs in PBL (E) and spleen (F) of indicated mice (E and F). D, ×40 and ×100 photomicrographs of sorted CD11b+Gr-1+ splenocytes from A10Tg mice. Flow cytometry plots and photomicrographs are representative of four independent experiments. Numbers on plots indicate the percentage of cells in the upper right (C) as well as upper right and lower right quadrants (E) and (F).

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of Ly6D (Fig. 4D). However, only 0.16% of A10Tg CLPs were Ly6D+. These findings indicate that hematopoietic alterations in A10Tg mice occur prior to the commitment of CLPs to the B cell lineage, and it explains the near absence of B cell precursors in the BM.

**ADAM10 alters myeloid but not LSK development**

To further determine the state at which A10 overexpression altered cell differentiation, we analyzed numerous subsets of LSKs and myeloid precursors. Multiple studies have shown that BM LSKs can be subdivided into various distinct populations based on CD34 and Flt3 expression: CD34<sup>−</sup>Flt3<sup>−</sup> long-term HSCs (LT-HSCs), CD34<sup>+</sup>Flt3<sup>−</sup> short-term HSCs (ST-HSCs), and finally CD34<sup>+</sup>Flt3<sup>+</sup> multipotent progenitor populations (MPPs) (36–38). We therefore analyzed these distinct populations within the LSK compartment in A10Tg mice.

Although the percentage of LSK cells was slightly lower in A10Tg BM (Fig. 5A), we did not observe any differences in the levels of LT-HSCs, ST-HSCs, or MPPs between LM and A10Tg mice (Fig. 5C). However, the percentage of myeloid progenitors (Lin<sup>−</sup>c-Kit<sup>hi</sup>sca<sup>−</sup>) in A10Tg mice was ~2-fold greater than the level in LM mice. It was previously shown that myeloid progenitors can also be subdivided into three distinct populations based on CD34 and low-affinity IgG FcRs (FcγRII/RIII). These include CD34<sup>+</sup>FcγRII/RIII<sup>lo</sup> CMPs, CD34<sup>+</sup>FcγRII/RIII<sup>hi</sup> granulocyte-macrophage progenitors (GMPs), and CD34<sup>+</sup>FcγRII/RIII<sup>lo</sup> megakaryocyte-erythroid progenitors (MEPs) (36). As shown in Fig. 5B, there is a striking difference in the myeloid compartment of A10Tg and LM BM. GMP and MEP populations are nearly absent from A10Tg mice. In addition, ~80% of the A10Tg myeloid progenitors are in the CMP stage compared with 31.6% in the LM. This finding may account for the systemic expansion of CD11b<sup>+</sup>Gr-1<sup>−</sup> MDSCs in A10Tg mice.

**ADAM10 overexpression alters hematopoiesis by dysregulating Notch signaling**

Although the mechanism by which ADAM10 regulates hematopoiesis remains ambiguous, recent studies have demonstrated its essential role in performing S2 cleavage of Notch receptors (see Discussion for model) during embryonic, thymocyte, and marginal

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**FIGURE 3.** ADAM10 alters hematopoiesis by a cell-autonomous intrinsic mechanism. Flow cytometric analysis of myeloid (A) and lymphocyte differentiation (B) in mixed BM chimeras generated as described in Materials and Methods 42 d after cell transfer. CD45.1<sup>+</sup>- and CD45.2<sup>+</sup>-gated cells differentiated from WT and A10Tg LSK BM, respectively. Data are representative of three independent experiments, except PBL data are representative of six independent experiments; numbers on dot plots indicate the percentage of CD45.1- or CD45.2-gated cells within boxes.

**FIGURE 4.** Overexpression of ADAM10 alters hematopoiesis prior to CLP commitment to the B cell lineage. Flow cytometric analysis of Lineage-negative (Lin<sup>−</sup>) versus CD19-labeled BM cells (A), B220 and IL-7R (IL7R) expression by Lin<sup>−</sup>CD19<sup>−</sup>-gated cells from A (B), c-Kit and sca-1 expression of Lin<sup>−</sup>CD19<sup>−</sup>B220<sup>−</sup>IL7R<sup>+</sup> cells from B (C), D, Expression of Ly6D by CLPs of LM and A10Tg mice. Numbers on dot plots and histograms indicate the percentage of gated cells. The cell lineage includes Ter-119<sup>−</sup>, CD11b<sup>−</sup>, Gr-1<sup>−</sup>, and CD3ε-positive cells. Plots are representative of three independent experiments. CLPs are defined as Lin<sup>−</sup>CD19<sup>−</sup>B220<sup>−</sup>IL7R<sup>+</sup>c-Kit<sup>hi</sup>sca-1<sup>−</sup>.
zone B cell development (3–5). Thus, to determine the effect of ADAM10 overexpression on Notch signaling in cell differentiation, we cultured purified LSK cells on OP9 stromal cells that either did not express Notch ligands (OP9-GFP) or expressed a primary Notch ligand, DL1 (OP9-DL1). The addition of IL-7 and Flt3 ligand promote LSK differentiation into T cells on OP9-DL1 cells and B cells on OP9-GFP cells (26). OP9-GFP cultures showed that although LM LSKs developed into B cells, A10Tg LSK cells only generated CD11b+Gr-1+ myeloid cells (Fig. 6A). OP9-DL1 cultures demonstrated that A10Tg LSK cells are capable of T cell differentiation (Fig. 6B). However, their development was delayed compared with LM LSK cells. This further supports the adverse effect of ADAM10 overexpression on development of B cell and thymocyte progenitors. Interestingly, high expression of DL1 on OP9-DL1 cells prevented myeloid expansion of A10Tg LSKs (Fig. 7A). This result suggests that ADAM10 overexpression may cause myeloid development by dysregulating Notch signaling in the absence of sufficient ligand.

To further test whether ADAM10 overexpression deregulates Notch signaling, we examined the effects of inhibiting Notch receptor cleavage in LM and A10Tg LSK cells. Inhibition of Notch signaling with the ADAM10 inhibitor (GI254023X) or the γ-secretase inhibitor (compound E) (14) in OP9-DL1 cultures prevented LM and A10Tg T cell development (Fig. 6C). In accordance with reports of ADAM10’s critical role in Notch S2 cleavage in other cell types (3–5, 8, 16, 39), this result indicates

**FIGURE 6.** Inhibition of γ-secretase or ADAM10 activity prevents Notch-dependent T cell development. A and B, Flow cytometric analysis of differentiated LSKs cocultured with OP9-GFP (A) or OP9-DL1 (B) stromal cells for 8, 17, and 29 d; representative of four independent experiments. C, T cell development of LSKs cocultured with OP9-DL1 cells for 29 d in the presence of a γ-secretase inhibitor, compound E, or an ADAM10 inhibitor, GI254023X; representative of two independent experiments.
that ADAM10 also mediates S2 cleavage in developing hematopoietic precursors. Intriguingly, although both inhibitors blocked Notch-dependent T cell development, their effects on development of myeloid and B lineage cells were distinct. ADAM10 inhibition in OP9-DL1 cultures resulted in B cell development of LM and A10Tg LSK cells. In addition, ADAM10 inhibition in OP9-GFP cultures not only resulted in enhanced LM B cell development but also it rescued B cell development of A10Tg cells and prevented myeloid differentiation.

In contrast to ADAM10 inhibition, GSI treatment of OP9-DL1 cultures caused myeloid development that was strikingly similar to myeloid differentiation of A10Tg cells in OP9-GFP cultures (Fig. 7). In addition, GSI treatment of LM OP9-GFP cultures had no effect on LSK development. This demonstrates that GSI treatment only directs myeloid development following Notch ligand binding and ADAM-mediated S2 cleavage, resulting in accumulation of the S2 cleavage product. Thus, both inhibition of the protease responsible for degrading the S2 product (γ-secretase) and overexpression of the protease that produces the S2 product (ADAM10) caused myeloid development. Collectively, these results indicate that ADAM10 overexpression may also direct myeloid development by generating excessive S2 product (see Fig. 8 model).

**FIGURE 7.** ADAM10 overexpression and γ-secretase inhibition disrupt Notch signaling and direct myeloid cell development. Flow cytometric analysis of LSK differentiation after 29 d of LSK coculture with OP9-DL1 (A) or OP9-GFP (B) stromal cells as described in Materials and Methods. Compound E, GI254023X, or control DMSO was added to media to inhibit γ-secretase or ADAM10 activity, respectively. DMSO-treated plots are representative of four independent experiments; plots of inhibitor-treated cultures are representative of two independent experiments. Numbers on plots indicate the percentage of gated live cells based on forward and side scatter.

**FIGURE 8.** A model of differential effects of Notch S2/S3 cleavage in hematopoietic differentiation. In the presence of ligand, the Notch receptor undergoes both S2 and S3 cleavage, resulting in two cleaved products and ultimately promoting T cell development (left panel). In the absence of ligand or in the case of blocked S2 cleavage, the Notch receptor remains intact and B cell development results (middle panel). However, S2 cleavage without S3 because of increased ADAM10 activity results in myelopoiesis (right panel).
Discussion

ADAM10-mediated S2 cleavage is required for the initiation of the canonical Notch signaling pathway. Accordingly, we hypothesized that ADAM10 activity would also regulate differentiation of early hematopoietic progenitors. Our experimental observations demonstrate that ADAM10 overexpression attenuates the development of thymocytes, aborts B2 cell development, and promotes expansion of functional MDSCs via a cell-intrinsic mechanism. Furthermore, the S2 and S3 cleavage products of Notch are shown to exert a differential effect on hematopoietic cell fate determination.

Although effects of Notch signaling in B and T cell lineage commitment have been extensively described, the activity of ADAM10 in cell differentiation and early B lineage commitment was uncharacterized previously. We demonstrate that overexpression of ADAM10 prevented B2 cell commitment from CLPs and that ADAM10 inhibitors in OP9 cultures enhanced B cell differentiation of LM and A10Tg LSKs. Thus, ADAM10 exerts adverse effects on B cell development. Further analysis of LSK, myeloid, and lymphocyte compartments demonstrates the stage at which ADAM10 overexpression alters hematopoiesis. Given that the LSK compartment is not affected, ADAM10 overexpression influences differentiation post-LSK subset differentiation but prior to mature lineage commitment (B cells, Ly6D+ CLPs, T cells [ETPs], CMPs, GMPs, and MEPs), which are dramatically affected in the A10Tg mice. The precise stage at which ADAM10 overexpression affects LSK commitment is likely to be very transient and remains to be determined. Although our data show a generalized overexpression of ADAM10 in BM, this overexpression is limited to B220+ cells. Thus, this transient phase may include a fraction of B220+ cells. Following differentiation of these B220+ cells into non-B cells, the transgene is no longer expressed, likely because the IgH enhancer is not used by other non-B cells.

The shift in favor of CMP development (Fig. 5B) could explain the robust accumulation of MDCs in the periphery. In addition, because CMPs have been shown to retain erythromyeloid potential, it is not surprising that despite the decreased MEP and GMP compartments, erythropoiesis remains unaffected. Furthermore, the presence of comparable NK cells (data not shown) and T cells in the periphery of A10Tg mice (Fig. 1D) suggests that although CLPs are unable to give rise to B cells, their ability to differentiate into ETPs and NK cells is retained.

It should be noted that upon lineage depletion, comparable BM cell numbers were recovered from both A10Tg and LM mice. Therefore, the percentage of ETPs obtained is reflective of absolute numbers of thymocyte precursors in the BM. Although peripheral T cell levels are comparable, the observed reduction in ETPs is nonetheless very intriguing and consistent with the diminished thymus seeding of A10Tg BM cells. This explains the reduced thymus size, relative inability of A10Tg thymocyte precursors to develop in mixed BM chimeras, and delayed T cell development in OP-DL1 cultures.

The classical model of hematopoiesis describes the initial dichotomous differentiation of LSKs into CLPs or CMPs. However, recent studies demonstrating that ETPs possess myeloid potential have challenged this model (21, 22). Additional studies demonstrated that B cell progenitors, including CLPs, also retain myeloid potential (40, 41), whereas other progenitors that lack T cell potential can develop into B cells or macrophages (42). For this reason, a myeloid-based model of LSK development is beginning to emerge (34, 43). In A10Tg mice, the moderate effects on thymocyte development in combination with the more pronounced effects on B lineage commitment and myeloid expansion indicate that B2 cells and expanded myeloid cells develop from common progenitor(s), whereas thymocytes may develop from a unique precursor.

The data presented are consistent with the hypothesis that excess ADAM10 causes ligand-independent S2 cleavage of Notch. It is possible that, in the absence of ligand, γ-secretase complex is not recruited to the cell membrane, thus leading to reduced S3 cleavage and accumulation of the S2 product. This would then result in enhanced myeloid development concurrent with diminished numbers of T cell progenitors. Given the high ligand expression levels in OP9-DL1 cells, we expected proper T cell differentiation in A10Tg LSKs as any excess S2 product would be processed by recruited γ-secretase. Indeed, even though T cell development in A10Tg LSK cells was delayed, it was comparable to LM at day 29. In addition, complete γ-secretase inhibition of LM and A10Tg OP9-DL1 cultures, a condition previously shown to cause S2 product accumulation (8, 16), mimicked myeloid development of A10Tg LSKs in the absence of ligand.

These findings support a model through which Notch cleavage mediates cell fate determination (Fig. 8). In the presence of ligand, the Notch receptor undergoes cleavage at both S2 and S3 sites, resulting in two products and ultimately promoting T cell development. In the absence of Notch ligand or in the case of blocked S2 cleavage, the Notch receptor remains intact and B cell development results. However, S2 cleavage without S3 cleavage, as observed during ADAM10 overexpression or γ-secretase inhibition, induces myelopoiesis and delayed T cell development. This is in agreement with other studies that have shown γ-secretase blockade to result in accumulation of the S2 product (8, 16), which could direct myeloid development. Indeed, diminished presenilin (PS)-dependent γ-secretase activity in PS1ΔE9/PS2ΔE9 mice results in myeloproliferative disease, characterized by accumulation of Mac-1/CD11bGr-1+ cells, causing splenomegaly (25).

The differential effects of S2 and S3 blockade on WT Notch signaling have significant implications for the treatment of Notch-related diseases. Many reports have proposed the use of GSIs for the treatment of T-ALL and B cell lymphoma (14, 44); however, our findings indicate that GSI treatment could cause MDSC expansion that would ultimately induce immunosuppression and enhance tumor growth. Thus, studies of GSI treatment in mice and clinical trials should include careful monitoring of myeloid cell development. This study indicates that pharmacologic blockade of S2 cleavage with ADAM10 inhibitors may be a more advantageous strategy.

Our data demonstrate that the proteolytic activity of ADAM10 regulates the lineage commitment of B2 cells and the expansion of functional MDSCs in a cell-intrinsic manner. Moreover, the data afford an in vivo model for further examination of MDSC expansion and MDSC-mediated immune suppression in the absence of confounding tumor-derived factors. Given that these factors also regulate immune responses (45), A10Tg animals provide a tool with which specific pathways leading to MDSC expansion may be elucidated in a controlled fashion. The singular expression of B1 cells in the absence of B2 cells likewise makes this mouse useful for the study of B1-mediated humoral immunity.

Furthermore, our observations support the developing myeloid-based model of hematopoiesis, leading us to propose a novel mechanism through which the Notch S2 and S3 cleavage products differentially regulate cell fate determination.

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Disclosures
The authors have no financial conflicts of interest.

References
**Figure S1** - Generation of A10Tg mice: (A) Schematic of the 7.5-kb XhoI injection fragment containing the murine ADAM10-HA cDNA and the murine H-2Kb promoter and IgH enhancer regulatory elements. (B) Southern blot analysis of genomic tail DNA from both founders (F) and their F2 progeny (F2a and F2b) digested with AccI and electrophoresed on a 0.9% agarose gel along with 1 kb DNA Ladder markers (M). The injection fragment shown in (A) was used as both probe and copy number control (5x and 25x). A10Tg lines 240 and 258 possess greater than 25 copies of the transgene, generating 1797 bp junction fragments (1612 bp + 185 bp) indicative of head-to-tail arrays, as well as 1270 bp and 4464 bp internal fragments. The founder of line 258 appears to be mosaic, identifiable as a transgenic by PCR only. (C) Flow cytometric analysis of pro/pre B cells (B220+Ig IgM-) and immature B cells (B220hiIgM+) in BM of littermate (LM) and A10Tg progeny. (D) ADAM10 surface expression by B220+IgM_cells and B220_IgM_cells shown in (C). Dot plots and histograms in (C,D) are representative of 6 independent experiments. (E) Western blot analysis of ADAM10 and _-actin protein levels in whole cell lysates of BM cells from indicated mice; ADAM10 pro-form (80 kDa) and ADAM10 mature form (60 kDa), representative of 3 independent experiments.

**Generation of ADAM10 Transgenic mice:** ADAM10 transgenic (A10Tg) mice were generated with the ADAM10-pHSE3’ transgene construct. ADAM10-pHSE3’ was produced by subcloning the murine ADAM10-HA cDNA from mADAM10-pcDNA3.1/Zeo (27) into the previously described pHSE3’vector (28), containing the murine H-2Kb promoter and IgH enhancer regulatory elements. Briefly, the ADAM10-HA cDNA was excised using BamHI/Sall and ligated into BamHI/XhoI cut pHSE3’.
ADAM10-pHSE3’ was amplified and analyzed by restriction endonuclease digestion and sequence analysis. A 7.5-kb XhoI fragment containing both cDNA and regulatory elements (Fig. S1A) was excised from ADAM10-pHSE3’ and injected into C57BL/6 (A10Tg line 240) or C57BL/6 x Balb/c (A10Tg line 258) embryos by the Virginia Commonwealth University Transgenic/Knockout Mouse Core. The resulting offspring were screened for the presence of the ADAM10-HA cDNA by PCR analysis of genomic tail DNA using ADAM10 cDNA sense (5’-CCGACAGTGTTAATTCTGCTCC-3’) and anti-sense (5’-TTCTTTCAGCCAGAGTTGTGCG-3’) primers. Amplification of DNA from A10Tg founders generated a 652-bp PCR product. Transgene integrity was verified and transgene copy number determined for both A10Tg lines by Southern blot analysis (Fig. S1B). Briefly, genomic tail DNA from both founders and their F2 progeny was digested with AccI and electrophoresed on a 0.9% agarose gel, and the injection fragment from ADAM10-pHSE3’ was used as both probe and copy number control. A10Tg line 258 was backcrossed with C57BL/6 mice for at least five generations.

**Western blots:** Whole cell lysates (30ug) of BM cells generated with lysis buffer containing 0.5% NP40 and protease inhibitors (Roche) were applied to SDS-PAGE and blotted on nitrocellulose membranes. Blots were probed with HRP-conjugated anti-mouse β-actin (Sigma-Aldrich) or unlabeled rabbit anti-ADAM10 (AnaSpec Inc.) followed by HRP-conjugated anti-rabbit IgG (Southern Biotech). Chemiluminescence was visualized with the Pico chemiluminescent kit (Pierce).