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Boosting apoptotic cell clearance by colonic epithelial cells attenuates inflammation *in vivo*

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SUMMARY

Few apoptotic corpses are seen even in tissues with high cellular turnover leading to the notion that the capacity for engulfment *in vivo* is vast. Whether corpse clearance can be enhanced *in vivo* for potential benefit is not known. In a colonic inflammation model, we noted that the expression of the phagocytic receptor *Bai1* was progressively downmodulated. Consistent with this, BAI1-deficient mice had more pronounced colitis and lower survival, with many uncleared apoptotic corpses and inflammatory cytokines within the colonic epithelium. When we engineered and tested transgenic mice overexpressing BAI1, these had fewer apoptotic cells, reduced inflammation, and attenuated disease. Boosting BAI1-mediated uptake by intestinal epithelial cells (rather than myeloid cells) was important in attenuating inflammation. A signaling-deficient *BAI1* transgene could not provide a similar benefit. Collectively, these complementary genetic approaches showed that cell clearance could be boosted *in vivo*, with potential to regulate tissue inflammation in specific contexts.

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AUTHOR CONTRIBUTIONS

C.S.L. designed and performed most of the experiments with guidance from K.S.R. K.K.P conducted the isolation of Th17 cells, lamina propria, and epithelial cells from colon. K.M.W and J.J.L performed testicular torsion experiments. I.J.J analyzed mesenteric lymph node (MLN) and spleen from mice. P.V. provided intellectual contributions in this study. C.S.L and K.S.R wrote the manuscript with input from co-authors.

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INTRODUCTION

Billions of cells are turned over on a daily basis in many tissues of the body as part of routine homeostasis (Arandjelovic and Ravichandran, 2015; Nagata et al., 2010). In addition, during infection and/or inflammation, additional cells undergo cell death, which includes cells native to the tissue as well as recruited immune cells such as neutrophils or lymphocytes (Nagata et al., 2010; Poon et al., 2014). The majority of cells that are turned over either homeostatically or during inflammation die by apoptosis (Nagata et al., 2010). The types of phagocytes that recognize and engulf apoptotic cells include professional phagocytes (such as macrophages and immature dendritic cells), as well as non-professional phagocytes (such as epithelial cells and fibroblasts) (Gregory and Pound, 2011; Poon et al., 2014). Even in tissues with high cellular turnover, very few apoptotic cells are seen under steady-state conditions. This has led to the general notion in the field that the capacity for engulfment *in vivo* is vast (Nagata et al., 2010; Ravichandran and Lorenz, 2007). However, interference with either the recognition of corpses or components of the phagocytic machinery (such as in genetically targeted mice) leads to a greater number of uncleared corpses in the tissues. Moreover, the uncleared apoptotic cells can become secondarily necrotic and this is linked to increased tissue inflammation, and a predisposition to autoimmunity (Hanayama et al., 2004; Scott et al., 2001). This suggests that the capacity for engulfment *in vivo* is perhaps not limitless. Also, the corollary, whether apoptotic cell clearance could be enhanced *in vivo*, and whether boosting the cell clearance in situations where uncleared corpses contribute to tissue inflammation has not been addressed.

Apoptotic cells expose ‘eat-me’ signals on their surface and phagocytes use specific receptors to recognize the eat-me signals on apoptotic cells (Ravichandran and Lorenz, 2007). A critical eat-me signal on apoptotic cells is the exposure of the lipid phosphatidylserine (PtdSer) (Fadok et al., 1992). PtdSer exposure as an eat-me marker is evolutionarily conserved from the nematode to humans (Fadok et al., 1992; Segawa and Nagata, 2015). While PtdSer is actively restricted to the inner leaflet of the plasma membrane in viable cells, it is exposed upon apoptosis-mediated as well as calcium-induced modes (Suzuki et al., 2013; Suzuki et al., 2010).

PtdSer on apoptotic cells can be recognized directly via receptors on phagocytes such as TIM-4, BAI1, and Stabilin-2 (Miyanishi et al., 2007; Park et al., 2007; Park et al., 2008). In addition, key receptors such as Mer-TK recognize PtdSer indirectly, via bridging molecules such as Gas-6 and Protein S, while $\alpha_v\beta_3$ integrins engage apoptotic corpses via the bridging molecule MFG-E8 (Lew et al., 2014; Rothlin et al., 2015; Scott et al., 2001; Zagorska et al., 2014). Relevant for this work, BAI1 is a member of the adhesion-family G-protein coupled receptors (GPCRs), with a long extracellular region, a 7transmembrane domain, and a long intracellular cytoplasmic tail. BAI1, via the thrombospondin repeats on its extracellular domain, directly engages PtdSer on apoptotic cells (Park et al., 2007). BAI1 signals intracellularly via the evolutionary conserved engulfment proteins ELMO1 and DOCK1 to activate the small GTPase RAC, and in turn, facilitate cytoskeletal rearrangements during corpse uptake (Brugnera et al., 2002; Gumienny et al., 2001).

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In this report, we first noted that endogenous *Bai1* expression progressively decreased over the course of DSS-induced colitis. Also, mice lacking BAI1 expression showed enhanced colonic inflammation, with increased uncleared corpses and more inflammatory cytokines. We then engineered transgenic mice overexpressing BAI1 to ask whether enhancing BAI1 expression can attenuate inflammatory colitis. These *BAI1* transgenic mice had reduced inflammatory cytokines in the colon, and overall attenuated disease. Via tissue specific overexpression of BAI1, boosting phagocytosis by epithelial cells of the colon was important for lowering this colonic inflammation. Conceptually, this provided a proof-of-concept that enhancing the engulfment machinery could be achieved *in vivo* and be of benefit in a specific disease context.

RESULTS

BAI1 deficiency affects apoptotic cell clearance after acute tissue injury in multiple tissues

To examine the role of BAI1 in apoptotic cell clearance *in vivo*, we generated mice with *Bai1* gene disruption (Figure S1A). The $Bai1^{-/-}$ mice appeared grossly normal, but bone marrow derived macrophages from $Bai1^{-/-}$ mice showed a defect in the engulfment of apoptotic targets as well as an increased release of inflammatory cytokines (Figure 1A). The lack of a global phenotype at steady state was likely due to the continued expression of *Bai1* homologous genes or the known redundancy in engulfment pathways, as gene deletion of individual engulfment genes often elicit a less pronounced global phenotype (Devitt et al., 2004; Elliott et al., 2010; Park et al., 2011; Scott et al., 2001).

Defects and inflammatory sequelae due to disruption of individual engulfment genes are often better revealed when mice are challenged with insults that induce apoptosis of a large number of cells within a tissue (Devitt et al., 2004; Elliott et al., 2010; Park et al., 2011; Scott et al., 2001). We tested this possibility in three different tissue contexts. First, when BAI1-deficient mice were challenged with dexamethasone to induce apoptosis in the thymus, the clearance of apoptotic thymocytes was compromised, with many remaining uncleared corpses (as shown by cleaved caspase 3 staining) (Figure 1B). Second, when we tested the BAI1-deficient mice in a model of testicular torsion, a form of ischemia-reperfusion injury where the apoptotic germ cells get cleared by Sertoli cells that line the seminiferous epithelium (Elliott et al., 2010; Park et al., 2011), the number of uncleared apoptotic corpses was greatly increased in mice lacking BAI1 (Figure 1C). Third, we assessed apoptotic cell clearance in a model of colonic inflammation. We chose the dextran sodium sulfate (DSS)-mediated colitis model, as this has been shown to mimic some aspects of the inflammatory features of human ulcerative colitis (Perse and Cerar, 2012) and apoptosis of colonic epithelial cells clearly contributes to DSS-induced colitis in this model (Qiu et al., 2011). The colonic tissue, both epithelial cells and the lamina propria fraction, primarily expressed *Bai1*, with minimal expression of the homologues *Bai2 and Bai3*, or the two other direct phosphatidylserine-binding receptors, *Timd4* and *Stab2* (Figure 1D and Figure S1B–S1D). DSS given orally via drinking water induced colitis over several days (Figure 1E). When we analyzed the presence of apoptotic cells within the colon after DSS treatment, the number of $TUNEL^{+}$ nuclei in the colonic epithelium of $Bai1^{-/-}$ mice was significantly increased relative to control mice (Figure 1F). When we stained for cleaved

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caspase3, an executioner caspase involved in apoptosis but not necroptosis, *Bai1*$^{-/-}$ mice showed significantly increased cleaved caspase 3^{+} cells in their colonic epithelium (Figure 1G), consistent with previous studies demonstrating that colitis induction after DSS treatment involves apoptosis (Gunther et al., 2011; Pasparakis and Vandenabeele, 2015; Qiu et al., 2011). Collectively, these data suggested that genetically removing BAI1 could lead to accumulation of uncleared apoptotic cells after induction of apoptosis in three different tissue contexts *in vivo*.

The deficiency of BAI1 augments acute colitis severity

We then assessed how the expression of endogenous *Bai1* might be modulated in the wild type mice after DSS treatment. Endogenous *Bai1* mRNA in colonic tissue and in the gut epithelial cells progressively decreased over time after DSS administration (Figure 2A and Figure S1E). Furthermore, when we tested HCT-116 colonic epithelial cells, *BAI1* mRNA in HCT-116 cells was also decreased by DSS treatment (Figure S1F). Moreover, in various transcriptomic analyses, *Bai11* expression as well as the downstream signaling intermediates *ELMO1, ELMO2, DOCK1*, and *RAC1* have been reported to be downmodulated in conditions of colonic inflammation in both mouse and human (Carey et al., 2008; Costello et al., 2005; Gao et al., 2013a; Kugathasan et al., 2008; Noble et al., 2008; Swan et al., 2013). These observations suggested that the complete loss of BAI1 in the gene-deleted mice, from the beginning of DSS administration, might contribute to greater to colonic inflammation, and we addressed this further.

When given 3% or 5% DSS in drinking water, the *Bai1*$^{-/-}$ mice showed greater weight loss at days 2, 3, 4, and 5 compared to control mice and also displayed an increase in disease severity index, which is a score that encompasses quantification of weight loss, bloody stools, and diarrhea (Figure 2B and 2C and Figure S1G and S1H). *Bai1*$^{-/-}$ mice showed decreased survival after 8 days of 5% DSS treatment with only 13% survival rate for *Bai1*$^{-/-}$ mice compared to 83% survival for co-housed controls (Figure 2D). Histologically, the DSS-treated *Bai1*$^{-/-}$ mice showed more destruction of the colonic epithelial structure, increased span of the inflamed region, and a thicker *muscularis* layer (Figure 2E). We also performed ‘continuous’ sections of the proximal region (which generally had more lesions) from multiple mice (~14 sections at 200 μm intervals per proximal region of mouse colon) and scored the number of TUNEL^{+} cells from these sections. TUNEL staining of the last six continuous sections of the proximal colon from two control and two *Bai1*$^{-/-}$ littermates are shown in Figure S2, with *Bai1*$^{-/-}$ mice showing much more TUNEL^{+} cells compared to their control littermates. Without DSS treatment, the *Bai1*$^{-/-}$ mice showed no increase in apoptotic cells in the colon and did not show any obvious colonic pathology compared to control mice (***data not shown***). The colon length (a disease parameter) was comparable in untreated control and *Bai1*$^{-/-}$ mice (at 8 and 40 weeks) (***data not shown***), but the DSS-treated *Bai1*$^{-/-}$ mice had significantly shorter colons (Figure S1I and S1J).

Consistent with prior studies that demonstrated acute DSS-induced colitis is minimally influenced by the adaptive immune system (Dieleman et al., 1994), the mesenteric lymph nodes and spleen did not show significant difference in lymphocyte numbers between *Bai1*$^{+/+}$ and *Bai1*$^{-/-}$ mice after DSS treatment (Figure 2F). Also, the CD4^{+}CD25^{+}Foxp3^{+}

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regulatory T (T_{reg}) cell subpopulations of the mesenteric lymph node and spleen were similar (Figure 2G). Furthermore, there was no difference in the T helper-17 (Th17) cell population (Figure 2H) isolated from the lamina propria of control and *Bai1*$^{-/-}$ mice. To minimize environmental influences that might affect colitis severity (Brinkman et al., 2013), the experimental animals and the appropriate littermate controls were always housed in the same cages and exposed to the same bedding, food, and water in all experiments. In addition, treatment of control and *Bai1*$^{-/-}$ littermates with an antibiotic cocktail (known to affect the bacterial population of the gut and the adaptive immune response (Diehl et al., 2013)) prior to initiation of DSS-induced colitis did not affect the enhanced colitis severity in BAI1-deficient mice (Figure 2I–2K). *Mertk*, a member of the TAM family of engulfment receptors continued to be expressed in the colonic tissue (Figure S1D), but was not able to overcome the loss of BAI1 under these DSS-treated conditions (note: expression of other two TAM members, *Tyro3* and *Axl*, was low). Collectively, these data suggested that mice lacking the phagocytic receptor BAI1 were more susceptible to acute injury to the colon in the context of DSS-induced colitis.

Transgenic overexpression of BAI1 attenuates DSS-induced colitis *in vivo*

We next asked whether forcibly increasing the expression of BAI1 would be beneficial in this colonic inflammation model. We engineered transgenic mice capable of conditionally overexpressing a HA-tagged human *BAI1* construct (*BAI1*^{Tg}). The human and mouse BAI1 are highly homologous and function comparably in the *in vitro* engulfment assays (Park et al., 2007), but using the cDNA for human *BAI1* as transgene allowed distinction from the endogenous mouse *Bai1*. A single copy of the wild type *BAI1* transgene was introduced via homologous recombination into the non-essential *Rosa26* locus in C57BL/6 mouse embryonic stem cells, and used to generate chimeric founder transgenic mice (*BAI1*$^{flox\text{-}STOP\text{-}flox}$). The expression of the *BAI1*^{Tg} was kept silent by a transcriptional and translational STOP cassette flanked by loxP sites, with cre-mediated deletion of the STOP cassette allowing expression of the HA-BAI1 and the bicistronic eGFP marker (Figure 3A). Initially, we expressed transgenic BAI1 globally by crossing the *BAI1*$^{flox\text{-}STOP\text{-}flox}$ mice to mice carrying *E2Acre*, which mediates ubiquitous deletion in most tissues (denoted as *E2A-cre, BAI1*^{Tg} mice) (Lakso et al., 1996).

We first confirmed the cre-dependent expression of transgenic human HA-tagged BAI1 and eGFP in the colon of the *E2A-cre, BAI1*^{Tg} mice (Figure 3B and 3C and Figure S3A and S3B). The peritoneal macrophages from *E2A-cre, BAI1*^{Tg} mice showed greater engulfment (about 18 %) compared to macrophages from control mice (Figure S3C). When the *BAI1*^{Tg} mice were tested in the testicular torsion model (Elliott et al., 2010; Park et al., 2011), the testis of *BAI1*^{Tg} mice had significant reduction in the number of apoptotic corpses compared to controls (Figure 3J). When tested in the dexamethasone-induced thymic apoptosis model, there was also a trend toward reduction in the number of apoptotic corpses in the *BAI1*^{Tg} mice but this did not achieve statistical significance due to the magnitude of apoptosis in this model (data not shown). When we assessed how the expression of transgenic human *BAI1* versus the endogenous mouse *Bai1* compared after DSS treatment, the transgenic human *BAI1* mRNA expression was unaffected in *E2A-cre, BAI1*^{Tg} mice by DSS administration, while the endogenous mouse *Bai1* mRNA was decreased as in control mice (Figure 3D and

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3E). This provided a system to test whether forcibly enhancing BAI1 expression could be beneficial in colonic inflammation.

We tested the *E2A-cre, BAI1*^{Tg} mice in the DSS colitis model, and made several observations: (i) The *E2A-cre, BAI1*^{Tg} mice had less weight loss after DSS treatment compared to controls (Figure 3F and Figure S3D and S3E, the controls being either *BAI1*^{Tg} mice that still had the STOP cassette, or mice carrying cre without the transgene); (ii) The *E2A-cre, BAI1*^{Tg} mice had an overall less disease severity index (Figure 3G and Figure S3F); (iii) The *E2A-cre, BAI1*^{Tg} mice had less colonic disease as measured by the colon length (Figure S3G and S3H); (iv) Histological analysis from multiple sections of DSS-treated mice showed that *E2A-cre, BAI1*^{Tg} mice had reduced relative area of inflamed colonic region, and thinner *muscularis* layer relative to controls, despite a similar baseline colonic architecture (Figure 3H); (v) The *E2A-cre, BAI1*^{Tg} mice had fewer TUNEL^{+} cells in their colonic epithelium compared to control littermates (Figure 3I). Collectively, these data suggested that increasing BAI1 expression could provide a ‘gain of function’ in an acute inflammation model of the colon.

Overexpression of a signaling deficient BAI1 mutant does not attenuate colitis severity

We asked whether this attenuated disease seen in response to increasing BAI1 expression was simply an effect of higher quantities of a membrane protein that can engage PtdSer or if it required BAI1 engagement of its downstream signaling components. BAI1 signals via the intracellular engulfment protein ELMO and its binding partner DOCK1 (either ELMO1 or ELMO2 depending on the cell type, with ELMO2 being more prominently expressed in epithelial cells) (Park et al., 2007). The ELMO-DOCK1 complex, in turn, leads to the activation of the small GTPase RAC, and the resulting cytoskeletal rearrangements within the phagocytes facilitate corpse internalization (Figure 4A) (Park et al., 2007). Of note, in unbiased transcriptome analysis, the expression of *ELMO2* and *DOCK1* have been shown to be lower in patients with inflammatory bowel disease (IBD) (Bjerrum et al., 2010; Burczynski et al., 2006; Costello et al., 2005) and in colitis models in animals (Schmidt et al., 2010). Mutation of three contiguous intracellular residues within the cytoplasmic tail of BAI1 (denoted as BAI1-AAA mutant) can abrogate the binding to ELMO and phagocytosis (Figure 4A) (Park et al., 2007). We engineered transgenic mice expressing the *BAI1-AAA* construct (essentially similar to the wild type *BAI1*^{Tg} mice above). For generating the BAI1-AAA transgenic founder mice, we used ES cell clones that could induce BAI1-AAA on the membrane equivalent to ES cells carrying the wild type *BAI1* transgene (data not shown). To induce mutant transgene expression, the *BAI1-AAA* mice were crossed to *E2A-cre* mice (*E2A-cre, BAI1-AAA*^{Tg}) and the BAI1-AAA expression was confirmed (Figure 4B and Figure S4A). When tested in the DSS inflammation model, the *E2A-cre, BAI1-AAA*^{Tg} mice did not show an attenuation of colitis severity compared to the control littermates, with comparable weight loss, disease severity index, colon length, and inflammatory region (Figure 4C–4F and Figure S4B–S4E). Also, there were comparable TUNEL^{+} nuclei in DSS-treated conditions in the control and *BAI1-AAA*^{Tg} mice (Figure 4G). These data demonstrated that BAI1 requires the engagement of downstream signaling components for attenuating colonic inflammation.

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BAI1 overexpression in the myeloid lineage fails to attenuate colitis severity

Since professional phagocytic cells of the myeloid lineage within the lamina propria are considered one of the key mediators in intestinal homeostasis and inflammation (Peterson and Artis, 2014), we first crossed the transgenic *BAI1*$^{flox\text{-}STOP\text{-}flox}$ mice with mice that drive *cre* expression under the lysozyme M promoter in multiple cells of myeloid lineage (*Lyz2-cre*) (Clausen et al., 1999). Total lamina propria, as well as macrophages from the lamina propria of colons (in addition to the peritoneal macrophages) from *Lyz2-cre, BAI1*^{Tg} mice confirmed myeloid expression of transgenic human BAI1 and/or eGFP (Figure 5A and Figure S5A and S5B). In *ex vivo* analysis, peritoneal macrophages from *Lyz2-cre, BAI1*^{Tg} mice showed enhanced the uptake of apoptotic cells (Figure S5C). *Lyz2-cre, BAI1*^{Tg} mice did not show a benefit in colitis after DSS treatment compared to control mice, as determined by the lack of a significant difference in the bodyweight change, disease severity index, colon length, and colonic inflammatory regions (Figure 5B–5D and Figure S5D–S5H). Thus, forcibly increasing BAI1 expression in myeloid cells is insufficient to reduce DSS-induced colonic inflammation. This prompted us to ask whether boosting BAI1 in other cell types would recapitulate the benefit seen in the *E2A-cre, BAI1*^{Tg} mice.

BAI1 overexpression in colonic epithelial cells is sufficient to attenuate colitis severity

Non-professional phagocytes, such as epithelial cells, can engulf apoptotic cells in other tissues including lung and mammary gland, and also produce anti-inflammatory mediators to dampen tissue inflammation (Bagci et al., 2014; Juncadella et al., 2013; Monks et al., 2008; Sandahl et al., 2010). Therefore, we asked whether boosting BAI1 expression within colonic epithelial cells contribute to dampening inflammation in the DSS colitis model. We crossed the transgenic *BAI1*$^{flox\text{-}STOP\text{-}flox}$ mice with mice carrying the cre under the villin 1 promoter (*Vil1-cre*) to induce the BAI1^{Tg} specifically in the intestinal epithelial cells (Madison et al., 2002). The *Vil1-cre, BAI1*^{Tg} mice (but not the littermate controls) showed specific expression of HA-BAI1 in the EpCAM-positive intestinal epithelial cells and colon (Figure 5E and Figure S6A–S6C), and transgenic BAI1 was undetectable in the EpCAM-negative cell types (Figure 5E and Figure S6C). Targeting BAI1 overexpression to colonic epithelial cells was sufficient to dampen inflammation in the DSS colitis model. Compared to control littermates, the *Vil1-cre, BAI1*^{Tg} mice showed relatively little weight loss, lower overall disease severity index, a thinner *muscularis* layer, increased colon length, and reduced inflammatory region (Figure 5F–5I and Figure S6D–S6F). Although we have not assessed BAI1 overexpression in other myeloid subtypes (such as dendritic cells), the data with *Vil1-cre, BAI1*^{Tg} mice implies that overexpression of BAI1 in colonic epithelial cells alone can reduce colitis severity.

We then asked how the improved disease parameters in *Vil1-cre, BAI1*^{Tg} mice correlate with apoptotic cell clearance within the colonic epithelium. First, the colonic epithelial cell line HCT-116 expressed *BAI1* mRNA and readily internalized apoptotic targets *in vitro*; since epithelial cells can also bind each other readily, the corpse internalization in this assay was confirmed by using as targets apoptotic epithelial cells labeled with a pH sensitive dye cypHer 5E (which fluoresces brighter after reaching the lower pH of the phagolysosome) (Figure 6A and 6B). Moreover, decreasing *BAI1* expression in HCT-116 cells via siRNA reduced the engulfment of apoptotic targets (Figure 6A and 6B), while overexpression of

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wild type BAI1, but not the signaling deficient BAI1-AAA mutant, enhanced uptake by HCT-116 cells (Figure 6C). Of note, silencing or overexpression of BAI1 did not affect the survival, apoptosis or proliferation rate of the colonic epithelial cells (data not shown). Another colonic epithelial cell line Caco-2 could also engulf apoptotic BEAS-2B or MLE-12 epithelial cells (Figure 6D).

We next asked whether the BAI1 overexpressing colonic epithelial cells in the *Vil1cre, BAI1^{Tg}* mice would show increased engulfment. We used a detection method that can score both the apoptotic cells and also the 'engulfed targets' within phagocytes. While the TUNEL assay *in vivo* detects DNase I cleavage within apoptotic nuclei (type I break) (Loo, 2011), the DNA derived from the engulfed targets undergoes DNase II-mediated cleavage (type II break) within the phagolysosomes (Figure 6E). We quantified both apoptotic (type I DNA cleavage) and engulfed cells (by monitoring type-II DNA cleavage) within the colonic epithelium (Evans and Aguilera, 2003). The colon sections of *Vil1-cre, BAI1^{Tg}* mice showed increased numbers of engulfed apoptotic cells (Figure 6F and 6G) with concomitantly fewer TUNEL-positive apoptotic nuclei in the same sections (Figure 6H and Figure S6G). In comparison, the control mice had more TUNEL positive apoptotic nuclei and relatively fewer engulfed cells (Figure 6F–6H). Co-staining for EpCAM and z-stack analysis of the sections showed that the engulfed targets were internalized, and resided within other EpCAM^{+} epithelial cells (Figure 6F and 6I and Figure S6H). These data suggested that increasing the expression of BAI1 within the intestinal epithelial cells could enhance cell clearance within the colonic epithelium, and in turn, attenuated colitis severity in this model.

BAI1 signaling modulates inflammatory cytokines in the colitis model

While the clearance of early stage apoptotic cells is anti-inflammatory, failure or delay in corpse clearance can lead these early apoptotic cells to become secondarily necrotic and also pro-inflammatory (Henson, 2005; Nagata et al., 2010). Since alarmins such as interleukin 1α (IL-1α) and IL-33 have been known to be released from damaged epithelial cells and linked to human colitis (Moussion et al., 2008; Suwara et al., 2014), we assessed their expression. Prior to the DSS treatment, the expression of inflammatory cytokine genes, *Il1a* and tumor necrosis factor (*Tnf*) were barely detectable in either the control or the *Bai1$^{-/-}$* mice, consistent with no obvious basal disease (Figure 7A). However, in the colonic tissue of *Bai1$^{-/-}$* mice, the qunatity of *Il1a* and *Tnf* were increased at day 5 of DSS treatment (Figure 7A and 7B). In addition, quantity of IL-33 mRNA and protein were significantly increased in *Bai1$^{-/-}$* mice (Figure 7A and 7B). The mRNA of other inflammatory cytokines, *Il6, Il12b*, and *Il22* (but not *Il17a*) were also increased in the *Bai1$^{-/-}$* mice compared to control mice (Figure S7A–S7D). In contrast, in mice globally overexpressing BAI1 (*E2A-cre, BAI1^{Tg}* mice) or specifically in the intestinal epithelial cells (*Vil1-cre, BAI1^{Tg}* mice), the mRNA for *Il1a* and *Tnf* were decreased compared to littermate controls, correlating with reduced colitis severity and fewer apoptotic cells in *BAI1^{Tg}* mice (Figure 7C and 7D). Also noteworthy is that mice transgenic for the mutant form of BAI1 (*BAI1-AAA^{Tg}* mice), did not display a reduction in *Il1a* and *Tnf* (Figure 7E).

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The increased inflammation seen in the mice lacking BAI1 could have occurred due to accumulation of corpses and/or the lack of signaling via BAI1 that dampens inflammation. However, this is not readily distinguishable in vivo, as corpse recognition, engulfment, and anti-inflammatory signaling are closely linked. Therefore, we tested the contribution of BAI1 *in vitro* in regulating inflammatory cytokine release. We measured the inflammatory cytokines in BAI1-silenced cells (HCT-116) and peritoneal macrophages of *Bai1*$^{-/-}$ mice after incubation with apoptotic cells. We found that silencing of BAI1 in HCT-116 resulted in higher amounts of the inflammatory cytokine *IL1A* and a modest reduction of the anti-inflammatory cytokine *TGFB1* and *Bai1*$^{-/-}$ mice in peritoneal macrophages also had increased amount of inflammatory cytokines (*Il1a, Il1b, Il33*, and *Tnf)* (Figure S7E–S7J). In addition, *in vitro*, treatment of HCT-116 cells with DSS could partially recapitulate the release of IL-1α as the cells became progressively more necrotic over a time course (Figure S7K–S7M). (Note: IL-33 was not detectable under these conditions from HCT-116 cells). These data suggest that the severity of colonic inflammation seen *in vivo* could have arisen from both the presence of uncleared apoptotic cells in the colonic tissue, as well as the reduction of anti-inflammatory signaling by BAI1.

DICUSSION

Collectively, the data presented in this work provide several key insights. First, it has often been assumed that the engulfment capacity of phagocytes *in vivo* is large or unlimited (Nagata et al., 2010). However, there are many inflammatory diseases where uncleared apoptotic cells are seen (Nagata et al., 2010), The data presented here suggest that the cell clearance capacity in a local tissue environment is in a balance, and that a ‘gain of function’ in cell clearance (and the associated dampening of inflammatory cytokines) is not only achievable, but might serve as a broad beneficial approach for certain inflammatory disorders. By generating transgenic mice with a gain of function in phagocytosis of apoptotic cells, and by targeting phagocytosis in specific cell-types, our data provide a genetic proof-of-concept that apoptotic cell clearance is genetically adjustable *in vivo*, and that enhancing the engulfment machinery is feasible *in vivo*. Our data also suggests that during colonic inflammation BAI1 contributes to regulating the severity of inflammation via the removal of apoptotic corpses and dampening of pro-inflammatory cytokines within the colonic tissue.

Second, while nutrient uptake and barrier function are the most commonly ascribed roles for gut epithelial cells, there is increasing appreciation for other functions of the epithelium, including coordinating communication and interaction with other cell types in the local gut environment (Devkota and Chang, 2013; Peterson and Artis, 2014; Spencer and Belkaid, 2012). Our data reveal a new functionally relevant role for colonic epithelial cells in engulfing their dying neighbors. Although the passive shedding of apoptotic colonic epithelial cells into lumen can occur, the relative importance of shedding versus clearance by neighboring epithelial cell in diseased and physiological states remains unclear.

Third, of note, it has been noted that the number of apoptotic colonic epithelial cells is considerably higher in colons of IBD patients and correlates with worse disease states (Di Sabatino et al., 2003; Hagiwara et al., 2002; Iwamoto et al., 1996). Our data here strongly

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suggest that the increased number of apoptotic cells and/or defects in cell clearance within the colon could be at least one additional contributing factor to colonic inflammation. This also adds a previously less appreciated viewpoint to the complex etiology of inflammatory bowel disease that affects millions of people worldwide (Loftus, 2004; Sartor, 2006). An interesting extrapolation from this work is that improving cell clearance by gut epithelial cells at the early stages of IBD, when the number of healthy epithelial cells capable of phagocytosis may be higher, could potentially help reduce the inflammatory responses seen in later stages of the disease. While recent antibody-based therapies targeting the inflammatory cytokines (such as TNF-α or IL-1) have proven effective in ameliorating disease symptoms in some patients (Colombel et al., 2010; Dassopoulos et al., 2013; Nielsen and Ainsworth, 2013; Vermeire et al., 2013), continued treatment predisposes these individuals to other opportunistic infections, as well as colon cancer (Targownik and Bernstein, 2013). Thus, approaches that pharmacologically augment cell clearance (which would simultaneously enhance the anti-inflammatory milieu associated with apoptotic cell clearance (Gregory and Pound, 2011; Lacy-Hulbert et al., 2007)) could represent a therapeutic modality worthy of further investigation.

EXPERIMENTAL PROCEDURES

For detailed description of all methods, please see Supplemental information.

Generation of *Bai1* deficient and *BAI1* transgenic mice

ES cells (GST_4419_G3) with a gene-trap mutation between exon 2 and 3 of *Bai1* were obtained from TIGM. To generate conditional *BAI1* transgenic mice, HA-tagged human *BAI1* cDNAs (encoding wild type *BAI1* and *BAI1-AAA* mutant) were inserted into the previously described CAG-STOP-eGFP-ROSA26TV (CTV) vector (Xiao et al., 2007). CTV-*BAI1* vectors were transfected into C57BL/6 embryonic stem cells (JM8A3) and screened for homologous recombination into *Rosa26* locus. Transgene encoded BAI1 and eGFP were confirmed in the ES cells *in vitro* via cre transfection. Selected ES clones were used to obtain transgenic mice. Tg-$BAI1^{flox\text{-}STOP\text{-}flox}$ mice were crossed with *E2A-cre* mice (Jackson laboratory), for global transgene expression (Lakso et al., 1996), *Lyz2-cre* mice (Jackson laboratory), (Clausen et al., 1999) for myeloid expression, *Vil1-cre* mice (Jackson laboratory) for intestinal epithelial cell expression (Madison et al., 2002). All animal experiments were performed according to protocols approved by the animal care and use committee (ACUC) at the University of Virginia.

DSS-induced colitis

Mice (littermates, 8 ~ 10 weeks old) were given 3 % or 5 % dextran sulfate sodium (DSS; MP Biomedicals) in drinking water for 5 or 8 days and analyzed on d5 or d8. Changes in body weight and disease severity index were checked daily. Disease severity was determined based on weight loss, blood in the stool, and stool consistency, as previously described (Owen et al., 2011). The total scores were calculated as follows: weight loss (0: < 1 %, 1: 1~5 %, 2: 6~10 %, 3: 11~15 %, 4: > 15 %), stool blood (0: negative, 2: positive, 4: gross bleeding), and stool consistency (0: normal, 2: loose stools, 4: diarrhea). All mice were randomly allocated to experimental groups and not recognized during the experiment.

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Staining for apoptotic and engulfed corpses

Mice were euthanized on day 5 after DSS treatment. Proximal colon region was cut by microtome at 200 μm intervals and TUNEL stained with the *In situ* cell death detection kit, TMR red (Roche) according to the manufacturer's instructions. Five random fields were captured per section for each mouse. Apoptotic indices from each field per mouse were integrated and shown as the mean ± s. e. m. Apoptotic cells and engulfed apoptotic cells were stained by labeling of DNA breaks due to DNase type I within apoptotic cells and DNase type II within phagolysosome using Apoptag ISOL dual fluorescence apoptosis detection kit (Millipore). Subsequently, the samples were incubated overnight with EpCAM antibody (APC conjugated; eBioscience; Catalog number 17-5791-80). The nuclei were counterstained with Hoechst 33342. The samples were analyzed using AxioImager Z2 equipped with Apotome for optical sectioning. About 10 fields of the proximal colon were captured for each mouse. Signals of DNA breaks induced by DNase type II were quantitatively analyzed by Image J (NIH) program. Engulfment index, the percentage of DNase type II versus the total nuclei signals (Hoechst 33342 staining) was calculated for each field. Engulfment indices from each field per mouse were integrated and depicted as mean ± s. e. m.

Statistical analysis

Sample numbers were determined based on optimal size sufficient statistical power. There were no inclusion/exclusion criteria for the analysis. Data are shown as means ± s.e.m. from multiple independent experiments. Student's two-tailed *t*-test was applied for analysis of statistical significance, defined as P values <0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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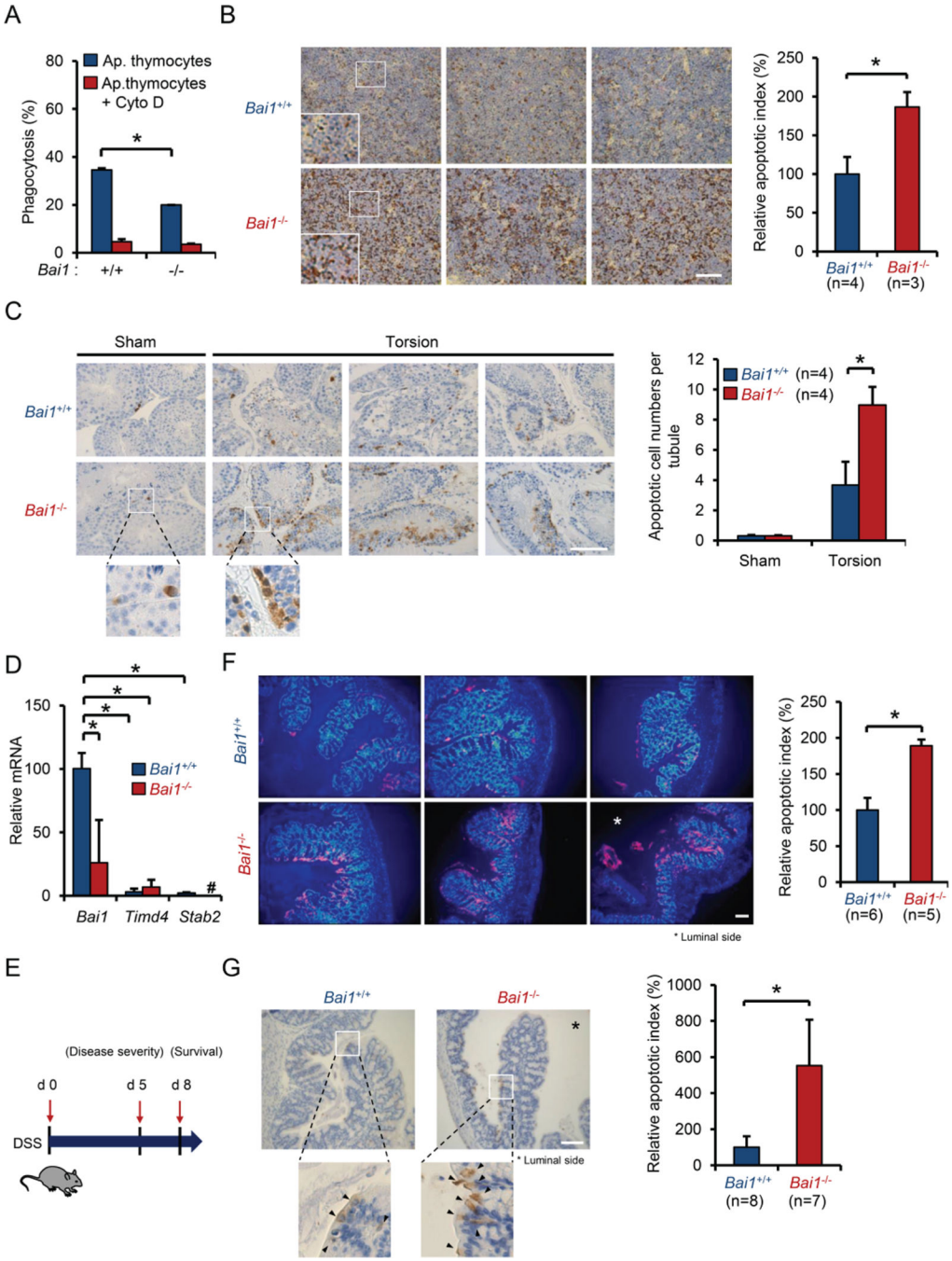

Figure 1. *Bai1*$^{-/-}$ mice have more uncleared corpses after apoptosis induction in three different tissues

(A) Engulfment of apoptotic thymocytes by BMDM from control and *Bai1*$^{-/-}$ mice were determined by flow cytometry. Cytochalasin D (CytoD) inhibits engulfment but not binding. (B) Cleaved caspase-3 staining of thymus from *Bai1*$^{+/+}$ and *Bai1*$^{-/-}$ mice after dexamethasone treatment (left) and its quantitation (right). (n=4 for *Bai1*$^{+/+}$ and n=3 for *Bai1*$^{-/-}$).

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(C) Cleaved caspase-3 staining of testes from *Bai1*$^{+/+}$ and *Bai1*$^{-/-}$ mice after sham treatment or testicular torsion (left) and its quantitation (right). (n=4; 4M for *Bai1*$^{+/+}$, n=4; 4M for *Bai1*$^{-/-}$).

(D) qPCR of *Bai1*, *Timd4*, and *Stab2* in colon of *Bai1*$^{+/+}$ (n=3) and *Bai1*$^{-/-}$ (n=3) mice (normalized to *Gapdh*). #, not detected.

(E) Schematic of the protocol for DSS-induced colitis and analysis.

(F) Apoptotic cells (TUNEL staining, red) in the proximal colonic region of *Bai1*$^{+/+}$ (n=6; 6M) and *Bai1*$^{-/-}$ (n=5; 5M) mice at day 5 after DSS (5 %) treatment. Hoechst 33342 was used to normalize the total nuclei among different sections. Apoptotic index of *Bai1*$^{+/+}$ was set to 100 %.

(G) Cleaved caspase-3 staining of proximal colon from *Bai1*$^{+/+}$ and *Bai1*$^{-/-}$ mice after DSS (5 %) treatment (left) and quantitated (right). (n=8; 8M for *Bai1*$^{+/+}$, n=7; 7M for *Bai1*$^{-/-}$). Data were from at least 2–3 independent experiments. Error bars indicate s.e.m. *P<0.05. M, male. Scale bars=50μm. See also Figures S1.

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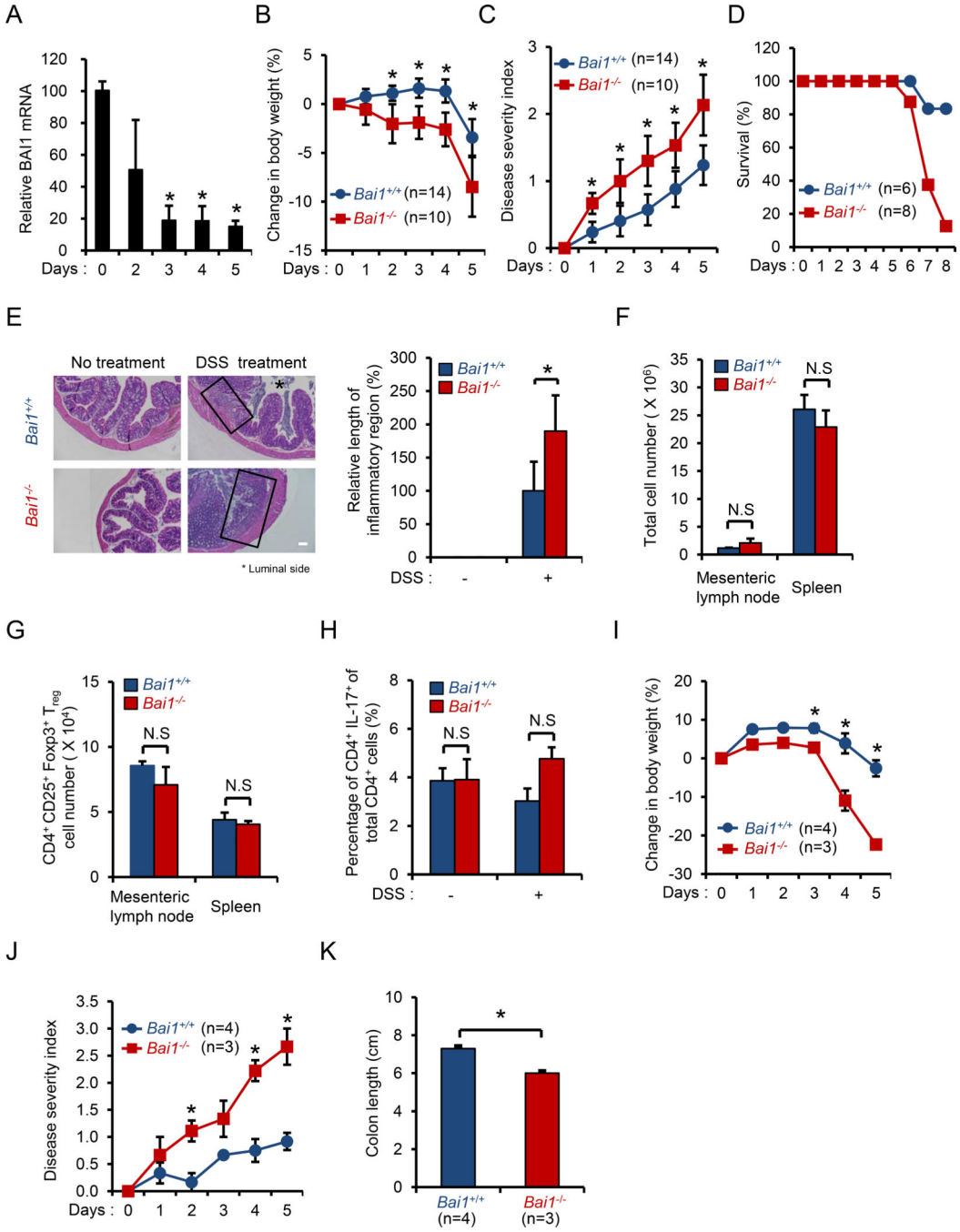

Figure 2. *Bai1*$^{-/-}$ mice are more susceptible to DSS-induced colitis

(A) Changes in *Bai1* mRNA in purified gut epithelial cells after DSS (5%) treatment (n=2; 2M per condition).

(B and C) Body weight and disease severity index of *Bai1*$^{+/+}$ (n=14; 14 M) and *Bai1*$^{-/-}$ (n=10; 10 M) mice after DSS (5%) treatment.

(D) Survival of *Bai1*$^{+/+}$ (n=6; 6M) and *Bai1*$^{-/-}$ (n=8; 8M) mice after DSS treatment.

(E) H&E staining of proximal colon from representative *Bai1*$^{+/+}$ and *Bai1*$^{-/-}$ mice before and after DSS (5 %) treatment (rectangle indicates inflamed region) and its quantitation (No

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treatment: n=5 for *Bai1*$^{+/+}$ and *Bai1*$^{-/-}$; DSS treatment: n=6; 6M for *Bai1*$^{+/+}$, n=5; 5M for *Bai1*$^{-/-}$). Scale bars=100μm.

(F, G) Total number of lymphocytes or CD4^{+}CD25^{+}Foxp3^{+} T_{reg} cells in mesenteric lymph node (MLN) and spleen of *Bai1*$^{+/+}$ (n=3; 3M) and *Bai1*$^{-/-}$ (n=4; 4M) mice after 5% DSS treatment.

(H) CD4^{+}IL-17^{+} Th17 cells from lamina propria of colon in *Bai1*$^{+/+}$ and *Bai1*$^{-/-}$ mice before and after DSS (5 %) treatment (No treatment: n=3; 3M for *Bai1*$^{+/+}$, n=3; 3M for *Bai1*$^{-/-}$; DSS treatment: n=2; 2M for *Bai1*$^{+/+}$, n=3; 3M for *Bai1*$^{-/-}$).

(I–K) Mice were given antibiotics for 4 weeks prior to DSS treatment, and the body weight (I), disease severity index (J), and colon length (K) were measured in *BAI1*$^{+/+}$ (n=4; 4M) and *BAI1*$^{-/-}$ (n=3; 3M) mice.

Data from at least two or three independent experiments were combined. Error bars indicate s.e.m. N.S, not significant. *P<0.05. M, male. F, female. See also Figures S1 and S2.

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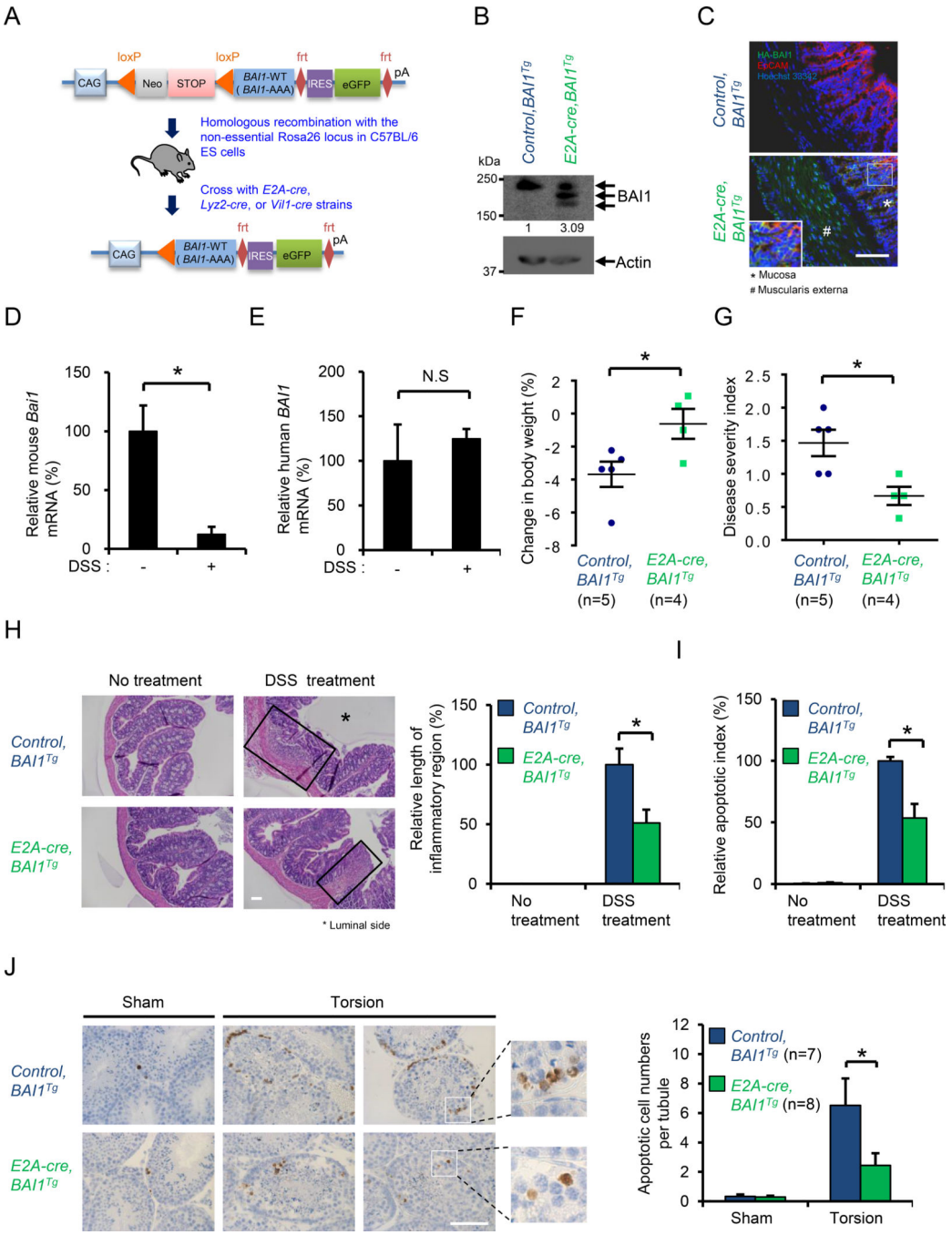

Figure 3. Transgenic overexpression of BAI1 attenuates acute colonic inflammation

(A) Schematic for generation of *BAI1*^{Tg} mice expressing human *BAI1*, via cre-mediated deletion of the STOP cassette preceding the transgenes. *E2A-cre* yielded global *BAI1*^{Tg} mice, while *Lyz2-cre* (myeloid) and *Vil1-cre* (intestinal epithelial cells) directed tissue specific *Bai1*^{Tg} expression.

(B) Detection of HA-BAI1 in the colon of control and *E2A-cre*, *BAI1*^{Tg} mice via immunoblotting. All three bands represent BAI1 (due to differential glycosylation).

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(C) Colon from control and *E2A-cre*, $BAI1^{Tg}$ mice were analyzed for HA-tag on transgenic BAI1 (green), the epithelial cell marker EpCAM (red) and Hoechst 33342 (blue). Scale bars=100 μm.

(D and E) Mouse *Bai1* mRNA and transgenic human *BAI1* mRNA in purified gut epithelial cells from *E2A-cre*, $BAI1^{Tg}$ mice after DSS (3%) treatment (No treatment: n=3, 3M; DSS: n=3, 3M).

(F and G) Change in body weight and disease severity index after DSS (3%) treatment in control (n=5; 5M) and *E2A-cre*, $BAI1^{Tg}$ (n=4; 4M) mice.

(H, I) H&E staining and apoptotic cell numbers in proximal colon from control and *E2A-cre*, $BAI1^{Tg}$ mice before and after DSS (3%) treatment (No treatment: n=5 for control, n=3 for *E2A-cre*, $BAI1^{Tg}$; DSS treatment: n=6; 6F for control, n=4; 4F for *E2A-cre*, $BAI1^{Tg}$). Scale bars=100μm.

(J) Cleaved caspase-3 (CC3) staining of testes from control and *E2A-cre*, $BAI1^{Tg}$ mice after sham treatment or testicular torsion (n=7; 7M for control, n=8; 8M for *E2A-cre*, $BAI1^{Tg}$). Scale bars=50μm.

Data are representative of at least two or three independent experiments. Error bars indicate s.e.m. *P<0.05. M, male. F, female. See also Figure S3.

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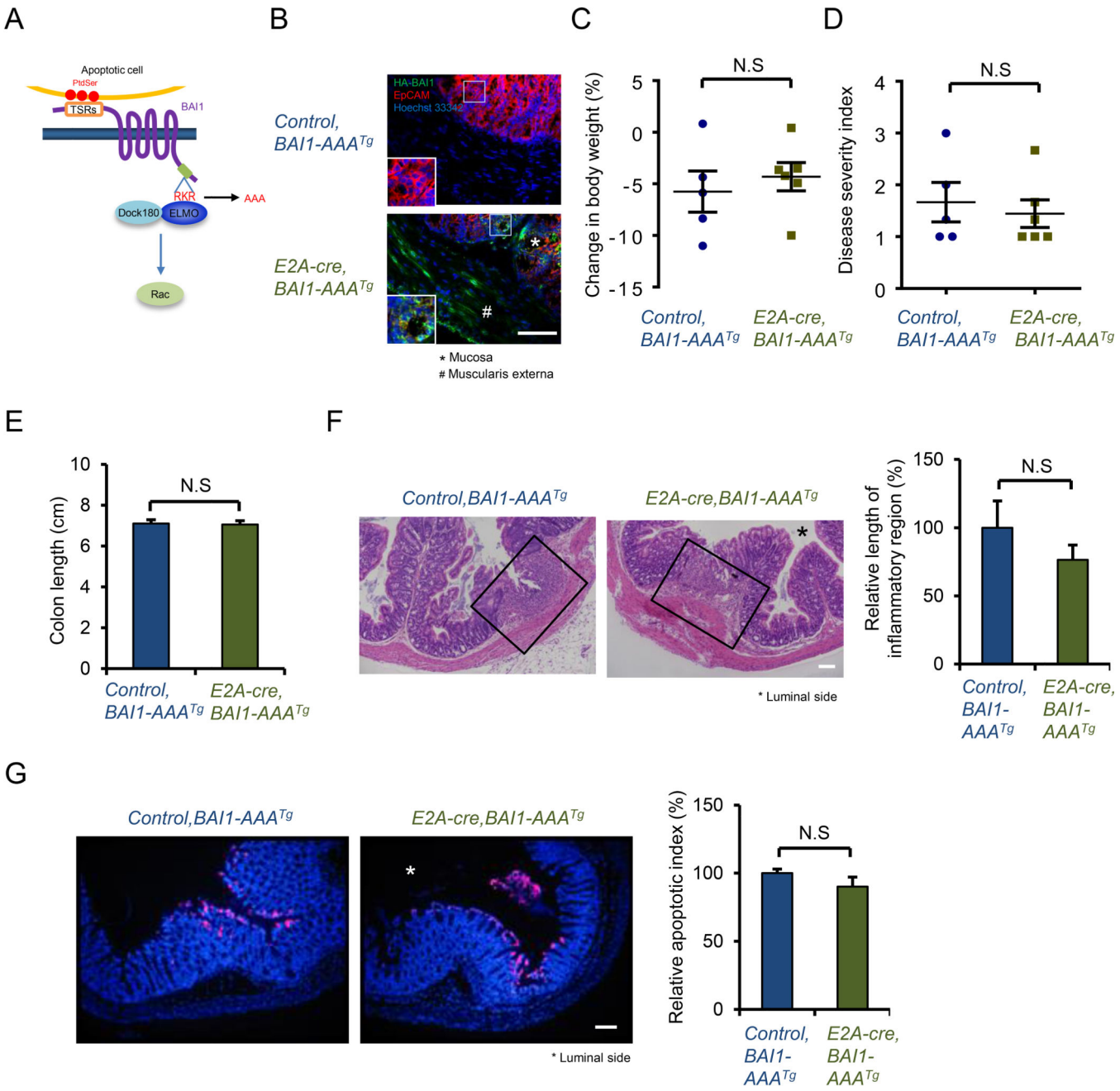

Figure 4. BAI1-ELMO dependent signaling is required for lowering colonic inflammation

(A) Schematic of wild type BAI1 with the AAA mutation indicated.

(B) Colons from control and *E2A-cre*, *BAI1-AAA*^{Tg} mice were stained for HA-BAI1 (green), EpCAM (red) and Hoechst 33342 (blue). Scale bars=100 μm.

(C and D) Changes in body weight and disease severity index after DSS (3%) treatment in control (n=5; 5M) and *E2A-cre*, *BAI1-AAA*^{Tg} (n=6; 6M) mice.

(E) Colon length in control (n=7; 7M) and *E2A-cre*, *BAI1-AAA*^{Tg} mice (n=11; 11M).

(F, G) H&E staining and apoptotic cell numbers within colons of control (n=3; 3M) and *E2A-cre*, *BAI1-AAA*^{Tg} (n=4; 4M) mice after DSS (3%) treatment. Scale bars=100μm.

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Data are representative of at least two or three independent experiments. Error bars indicate s.e.m. N.S, not significant. M, male. See also Figure S4.

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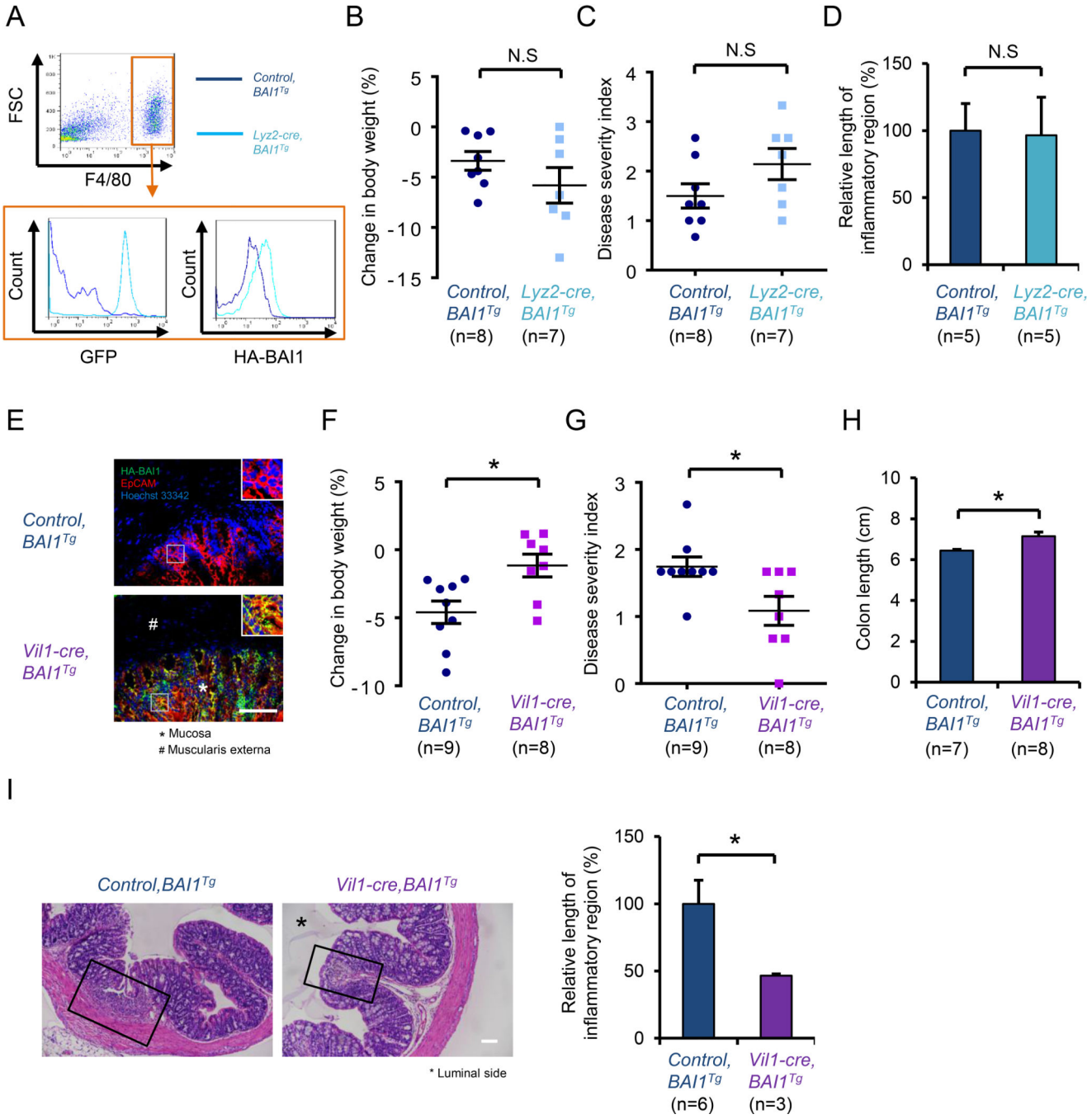

Figure 5. BAI1 overexpression in colonic epithelial cells is sufficient to dampen DSS-induced colitis

(A) Analysis of eGFP and HA-BAI1 expression in F4/80 positive peritoneal macrophages of control and *Lyz2-cre, BAI1^{Tg}*.

(B, C) Change in body weight and disease severity index after DSS (3%) treatment in control (n=8; 8M) and *Lyz2-cre, BAI1^{Tg}* (n=7; 7M) mice.

(D) Quantitation of inflamed regions in proximal colon of control (n=5; 5M) and *Lyz2-cre, BAI1^{Tg}* (n=5; 5M) mice after DSS (3 %) treatment.

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(E) Colons of control and *Vil1-cre, BAI1*^{Tg} mice after staining for HA-BAI1 (green), EpCAM (red) and Hoechst 33342 (blue). Scale bars=100 μm.
(F and G) Change in body weight and disease severity index after DSS (3 %) treatment in control (n=9; 9M) and *Vil1-cre, BAI1*^{Tg} (n=8; 8M) mice.
(H) Colon length in control (n=7; 7M) and *Vil1-cre, BAI1*^{Tg} (n=8; 8M) mice after DSS (3%) treatment.
(I) H&E staining of colon from control (n=6; 6F) and *Vil1-cre, BAI1*^{Tg} mice (n=3; 3F) after DSS (3%) treatment Scale bars=100μm.
Data from at least two or three independent experiments were combined. Error bars indicate s.e.m. N.S, not significant. *P<0.05. M, male. F, female. See also Figures S5 and S6.

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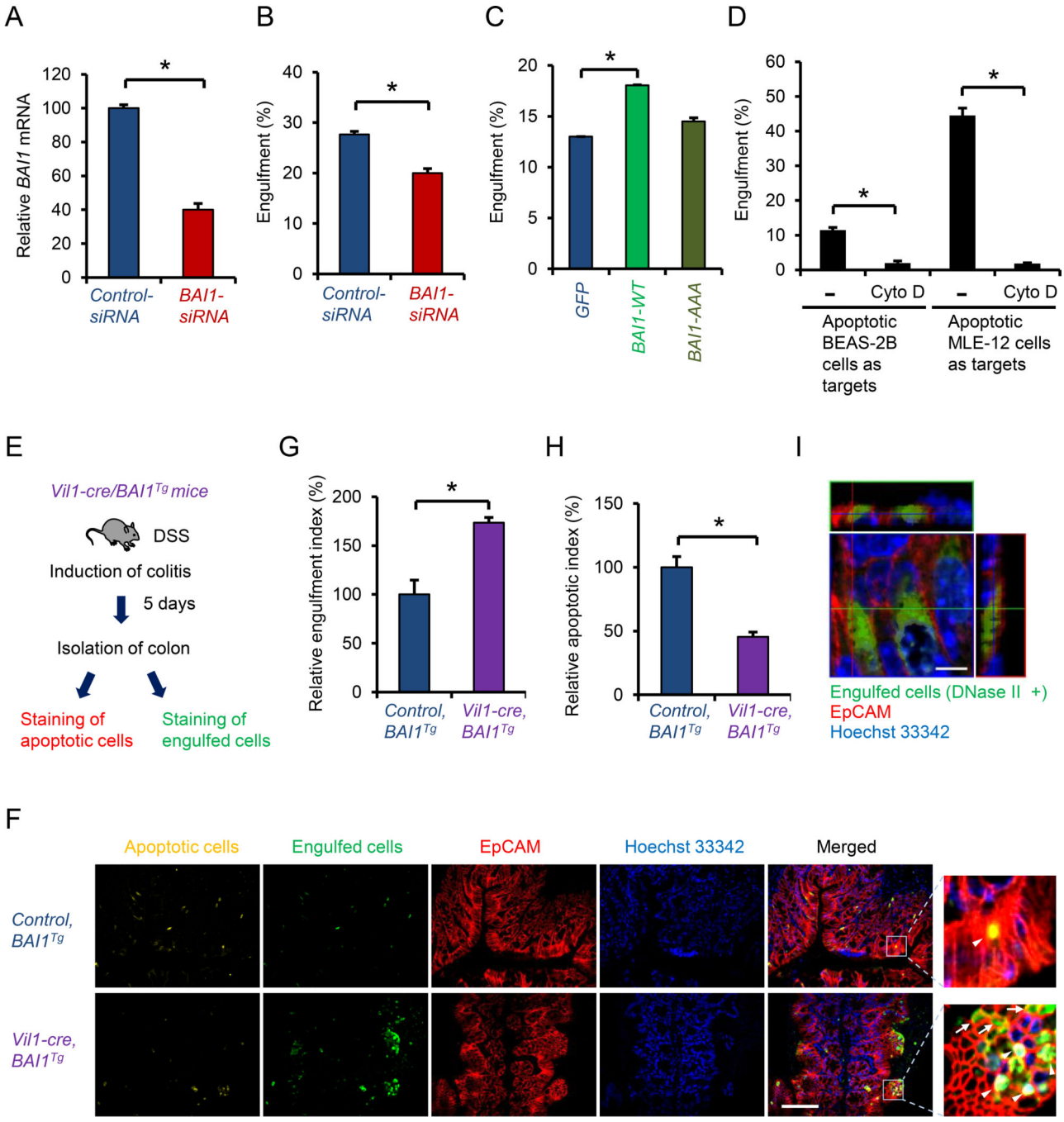

Figure 6. Colonic epithelial cells engulf apoptotic cells within the colon

(A) Silencing of *BAI1* by siRNA in colonic epithelial cell line HCT-116 (normalized to *HPRT1*).

(B) HCT-116 cells transfected with *control-siRNA* and *BAI1-siRNA* for 48 hours were assessed for engulfment of apoptotic MLE-12 cells labeled with CypHer5E.

(C) HCT-116 cells transfected with *GFP, BAI1-WT*, and *BAI1-AAA* were assessed for engulfment of apoptotic MLE-12 cells labeled with CypHer5E.

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(D) Engulfment by Caco-2 cells was assessed with apoptotic BEAS-2B or apoptotic MLE-12 cells labeled with CypHer5E. Cytochalasin D (blocks uptake but not binding), was used to ensure that the signal is due to corpse engulfment.
(E) Schematic of staining of apoptotic cells (TUNEL, type I DNA break) and engulfed cells (DNase II-mediated type II break within phagocytes) after DSS (3 %) treatment.
(F and G) Analysis of apoptotic (DNase type I cleavage, yellow) and engulfed corpses (DNase type II cleavage, green) within the proximal colon of control (n=6; 6F) and *Vil1-cre, BAI1*^{Tg} (n=3; 3F) mice after DSS (3 %) treatment. In the enlarged images, the arrows indicate signal for engulfed corpses within EpCAM^{+} epithelial cells, and arrow heads indicate double positive cells (signal for apoptotic and engulfed). Hoechst33342 used to normalize between sections; the number of engulfed cells in control animals was set to 100 %. Scale bars=100 μm.
(H) Analysis of apoptotic cell numbers in proximal colon of control (n=6; 6F) and *Vil1-cre, BAI1*^{Tg} (n=3; 3F) mice after DSS (3 %) treatment.
(I) z-stack image of engulfed cells (DNase II positive, green) within colonic epithelial cells. Scale bars=5 μm.
Data from at least 2–3 independent experiments were combined. Error bars indicate s.e.m. *P<0.05. F, female. See also Figure S6.

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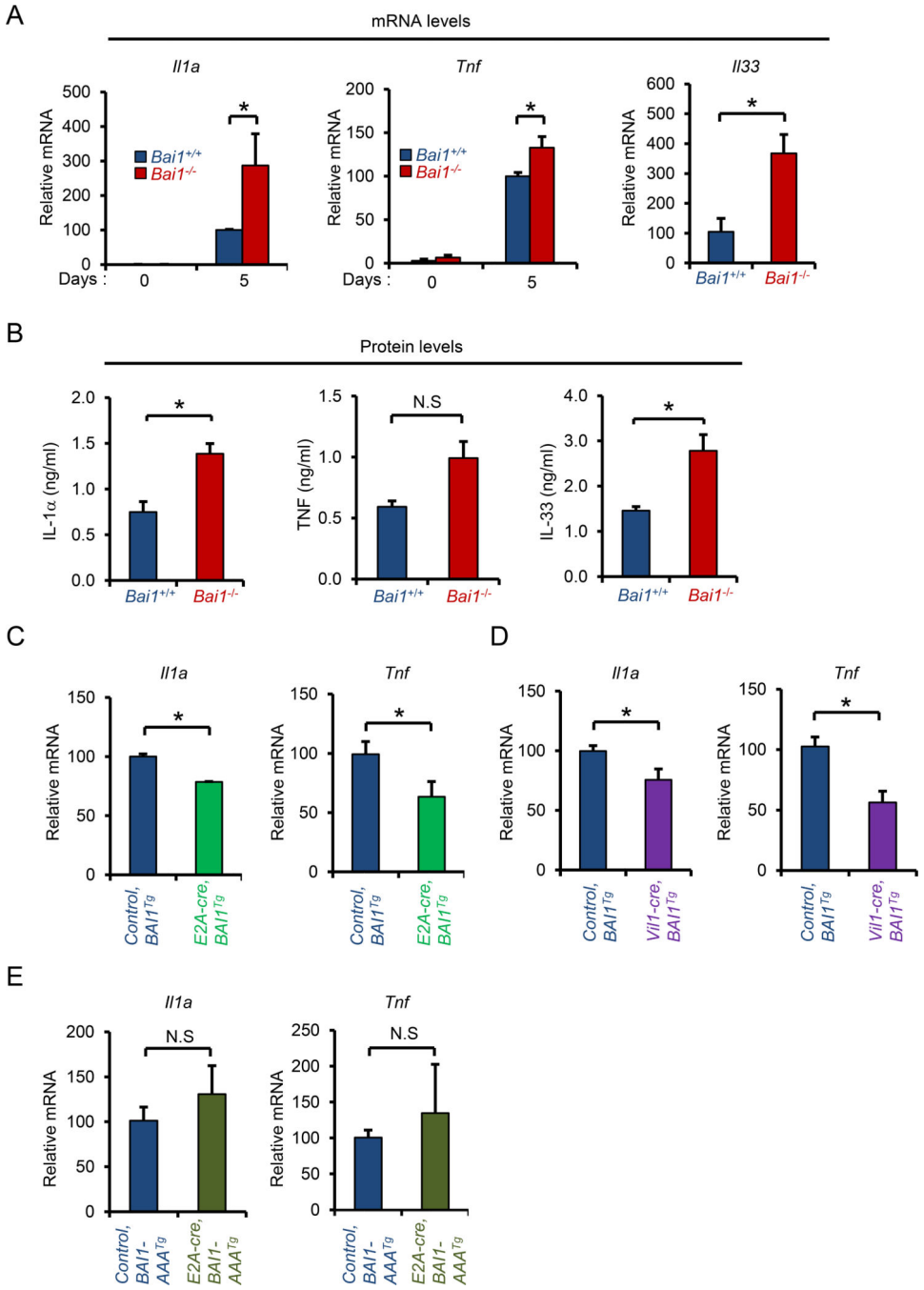

Figure 7. Inflammatory cytokine profiles correlate with diminished or enhanced apoptotic cell clearance

(A) qPCR of *Il1a, Tnf*, and *Il33* in colon of *Bai1*$^{+/+}$ and *Bai1*$^{-/-}$ (No treatment: n=5 for *Bai1*$^{+/+}$, n=4 for *Bai1*$^{-/-}$; DSS: n=4; 4M for *Bai1*$^{+/+}$, n=3; 3M for the *Bai1*$^{-/-}$) mice at day 0 and day 5 after DSS (normalized to *Gapdh*).

(B) ELISA for IL-1α, TNF, and IL-33 in colon of *Bai1*$^{+/+}$ and *Bai1*$^{-/-}$ mice at day 5 after treatment (DSS: n=3; 3F for *Bai1*$^{+/+}$, n=3; 3F for the *Bai1*$^{-/-}$).

(C–E) qPCR of *Il1a* and *Tnf* in colon of *E2A-cre, BAI1*^{Tg} (n=3; 3M for control, n=3; 3M,for *E2A-cre, BAI1*^{Tg}), *Vil1-cre, BAI1*^{Tg} (n=3; 3M for control, n=3; 3M for *Vil1-cre, BAI1*^{Tg}),

and *E2A-cre, BAI1-AAA*^{Tg} (n=2; 2M for control, n=5; 5M for *E2A-cre, BAI1-AAA*^{Tg}) mice at day 5 after DSS treatment (normalized to *Gapdh*).

Data from at least 2–3 independent experiments were combined. Error bars indicate s.e.m. N.S, not significant. *P<0.05. M, male. F, female. See also Figure S7.

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