Sterile inflammation enhances ECM degradation in integrin β1 KO embryonic skin

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Sterile Inflammation Enhances ECM Degradation in Integrin β1 KO Embryonic Skin

Highlights
- Loss of integrin β1 in embryonic epidermis elicits a sterile inflammatory response
- Tissue-resident and monocyte-derived macrophages remodel the ECM
- Activation of TGF-β1 by integrin β6 drives the inflammatory response
- Targeting inflammation with NSAIDs, TGF-β, and NF-κB inhibitors rescues ECM defects

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In Brief
Kurbet et al. demonstrate a link between macrophage recruitment and ECM disorganization in β1 integrin KO embryonic skin. The inflammatory response is driven by TGF-β1 activation through upregulated integrin β6. Pharmacological inhibition of the inflammatory response by treating KO embryos with NSAIDs, TGF-β, and NF-κB inhibitors rescues ECM disorganization.
Sterile Inflammation Enhances ECM Degradation in Integrin β1 KO Embryonic Skin

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SUMMARY

Epidermal knockout of integrin β1 results in complete disorganization of the basement membrane (BM), resulting in neonatal lethality. Here, we report that this disorganization is exacerbated by an early embryonic inflammatory response involving the recruitment of tissue-resident and monocyte-derived macrophages to the dermal-epidermal junction, associated with increased matrix metalloproteinase activity. Remarkably, the skin barrier in the integrin β1 knockout animals is intact, suggesting that this inflammatory response is initiated in a sterile environment. We demonstrate that the molecular mechanism involves de novo expression of integrin αvβ6 in the basal epidermal cells, which activates a TGF-β1 driven inflammatory cascade resulting in upregulation of dermal NF-κB in a Tenascin C-dependent manner. Importantly, treatment of β1 KO embryos in utero with small molecule inhibitors of TGF-βR1 and NF-κB results in marked rescue of the BM defects and amelioration of immune response, revealing an unconventional immuno-protective role for integrin β1 during BM remodeling.

INTRODUCTION

Integrity at the dermal-epidermal junction (DEJ) is critical to maintaining skin homeostasis and barrier function. Basal keratinocytes attach to the underlying extracellular matrix (ECM) via heterodimeric transmembrane integrin receptors, which activates bidirectional signaling cascades integral to cellular processes such as proliferation, migration, and differentiation (Hegde and Raghavan, 2013; Hynes, 2002; Margadant et al., 2010). Epidermal basal keratinocytes are separated from the underlying dermis by a contiguous basement membrane (BM) made up of an ordered array of ECM molecules, which serve important structural and signaling functions (Blanpain and Fuchs, 2009; Breitkreutz et al., 2013). The major proteins that make up the epithelial BM are collagen IV and laminin 332 (laminin 5), which require the expression of αβ1 integrins in the basal keratinocytes to organize (Brakebusch et al., 2000; deHart et al., 2003; DiPersio et al., 1997; Raghavan et al., 2000). It is now well appreciated that the ECM is not only important for structural rigidity, but is also a major determinant of cellular behavior such as cytoskeletal reorganization, integrin signaling, and growth factor signaling (Hynes, 2009). It is a dynamic reservoir for several growth factors and cytokines released during development or in response to injury (Hynes, 2009). ECM-remodeling enzymes such as matrix metalloproteinases (MMP) are key mediators of ECM plasticity (Visse and Nagase, 2003). MMP-dependent remodeling of ECM is dysregulated in pathological conditions like fibrosis and cancer (Bonnans et al., 2014; Cox and Erler, 2011).

Apart from the physical barrier formed by the terminally differentiated layers and underlying BM, it is well appreciated that the innate and adaptive immune systems form a formidable barrier to breaches and pathogenic infection throughout the lifetime of an organism. The main classes of innate immune cells in the skin are Langerhans cells, dendritic cells, tissue-resident macrophages, and circulating monocytes (Pasparakis et al., 2014). Precursors of dendritic Langerhans cells are early infiltrates of embryonic skin, and have been recently shown to be seeded from the embryonic yolk sac as well as fetal liver (Hoeffel et al., 2012). However, how these precursors and other immune cells contribute to immune surveillance during embryonic skin development is not completely understood. Most of our understanding of fetal immune development at the body’s interface with the surrounding is derived from studies of the gastrointestinal system (McElroy and Weitkamp, 2011; Warner and Hamvas, 2015).

While the events that underlie a pathogen-driven inflammatory response have been elucidated, less is known about how inflammation is initiated by damage-associated molecular patterns (DAMPs). This is also known as sterile inflammation,
since it occurs in the absence of any pathogens (Rock et al., 2010). A defining feature of sterile inflammation is that it can often result in chronic inflammatory diseases. For example, long-term inhalation of particulates like asbestos can result in the chronic activation of alveolar macrophages, resulting in pulmonary fibrosis (Mossman and Chung, 1998). Likewise, gout has been associated with accumulation of urate crystals in the joints, resulting in extensive tissue damage due to an acute inflammatory response mediated by neutrophils (Chen and Nuñez, 2010). In ischemic-reperfusion injury commonly seen in strokes and acute myocardial infarctions, the restoration of blood flow paradoxically causes further tissue damage by neutrophils due to release of DAMPs like reactive oxygen species (ROS) from dying cells (Eltzschig and Eckle, 2011). Endogenous antigens can also serve as DAMPs to elicit a sterile inflammatory response mediated by Toll-like receptors (TLRs) (Medzhitov, 2008; Srikrishna and Freeze, 2009). Studies conducted in the context of chronic inflammation have revealed the important role played by ECM-derived DAMPs in eliciting an innate immune response that potentiates adaptive immunity (Midwood and Orend, 2009). ECM components, such as hyaluronic acid, heparan sulfate proteoglycan (HSPG), and Tenascin C (TNC) are known to act as proinflammatory DAMPs and can trigger inflammatory responses (Adair-Kirk and Senior, 2008; Goh et al., 2010). TNC is expressed subepidermally in injured skin and precedes inflammation (Chiquet-Ehrismann and Chiquet, 2003), indicating the role ECM proteins and their degradation products can play as potent inducers of inflammation. Importantly, TNC expression is implicated in pathogenesis of autoimmune diseases like rheumatoid arthritis and Crohn’s disease (Goh et al., 2010).

There is a critical need to understand the earliest events that set up sterile inflammation, which have remained elusive thus far. This is important not only to understand the progression of chronic inflammatory diseases, but also to design effective therapeutic strategies for the same. These efforts have been stymied to a large extent by the lack of appropriate model systems.

In the present report, we have utilized the conditional integrin β1 knockout (KO) mouse model to better understand the role of sterile inflammation in remodeling the ECM during embryonic development. We report a unique inflammatory response observed due to epidermis-specific loss of integrin β1 involving aberrant recruitment of innate and adaptive immune cells, which occurs in the presence of an otherwise intact skin barrier. This immune response at the DEJ was found to be driven by TGF-β and MMP activity at the DEJ Leading to a Progressive Loss of ECM Organization

RESULTS

Loss of β1 in the Skin Epidermis Results in the Upregulation of Proinflammatory Genes and Increased MMP Activity at the DEJ Leading to a Progressive Loss of ECM Organization

As previously reported, the conditional loss of β1 integrin (β1 KO) in the epidermis leads to loss of BM organization and impaired hair follicular morphogenesis (Raghavan et al., 2000). However, several integrin-dependent and independent molecular events in these animals have not been investigated. We followed the loss of BM organization in the developing integrin β1 KO embryos. Starting as early as embryonic day E16.5 there was a marked disorganization of laminin 5 at the DEJ in β1 KO animals (Figures 1A and 1B). This disorganization got progressively worse by E18.5, resulting in a complete separation between the epidermis and underlying dermis (Figures 1C–1F). We measured the expression of other ECM components like collagen IV, entactin, fibronectin, and HSPG and found them to be similarly disorganized (Figures S1A–S1H). Additionally, we found keratin 6 (K6), a known stress-response keratin (Lassard et al., 2013; Mazzalupo et al., 2003), to be highly upregulated in the β1 KO epidermis (Figures S1I and S1J), suggesting that loss of integrin β1 in the epidermis could result in adhesion defects eliciting a stress response. The BM disruption observed in β1 KO skin samples is much more severe when compared to related integrin KO models such as integrin α6 or β4 KO (Dowling et al., 1996; Georges-Labouesse et al., 1996; Raghavan et al., 2000). This pointed to potentially additional molecular pathways contributing to the severity of the adhesion-defect phenotype. We performed next-generation sequencing (NGS) on RNA isolated from E18.5 wild-type (WT) and β1 KO skin and clustered enriched genes based on gene ontology (GO). Interestingly, most of the GO terms involved response to physiological processes such as inflammation, chemotaxis, and wound healing (Figure 1G). We validated some of the highly upregulated genes (Table 1) by real-time qPCR and subsequently classified them as inflammatory cytokines (IL-18, IL-1β, IL-33, and IL-13), chemotactic chemokines (CCL8, CCL20, CCL22, CXCL5, and CXCL16), and metalloproteinas in response to wounding (MMP 9 and 13) (Figure 1H). The proinflammatory cytokatypes enriched in our analyses belong to IL-1 family. These cytokines are predominantly produced by monocytes, dendritic cells, and macrophages and are central to their activation and proliferation (Arango Duque and Descoteaux, 2014). Chemokines such as CCL8 and CXCL5 are known to be important for homing and trafficking of inflammatory monocyte-derived macrophages (Mantovani et al., 2004). Notably, macrophage-derived CXCL16 is integral to the activation of T lymphocytes (Mantovani et al., 2004). This suggested that the microenvironment of embryonic integrin β1 KO skin is flush with chemotactic and proinflammatory factors which attract innate and adaptive immune cells and potentially sustain their response.

Since we found that various MMP RNAs were upregulated in the integrin β1 KO skin (Table 1), and these have been shown to be potent remodelers of the BM, we further analyzed if they were in the active form or present as proenzymes. We performed in situ zymography over progressive embryonic stages to assay MMP activity at the DEJ in β1 KO mice. The progressively
increasing ECM degradation (Figures 1B, 1D, 1F, and S1B) was closely matched by higher MMP activity from E16.5 to E18.5 (Figures 1J, 1L, and 1N). Meanwhile, WT littermates had negligible MMP activity at the DEJ (Figures 1I, 1K, and 1M). Interestingly, there were no significant changes in protein or gene expression of ECM components in the b1 KO animals (Figures S1K and S1L). Collectively, these findings suggest that integrin b1 KO embryos generate an inflammatory wound-response signature and precipitate matrix remodeling at the DEJ, resulting in loss of BM organization.

The Immune Infiltrate Recruited to the BM in Developing Integrin b1 KO Skin Comprises of Tissue-Resident Macrophages, Monocyte-Derived Macrophages, and CD3+ T Lymphocytes

We next investigated if the increased expression of chemokines and cytokines in the integrin b1 KO was associated with increased recruitment of immune cells during embryonic development (E16.5 to E18.5). Compared to WT littermate controls, we observed a distinct increase in the recruitment of both F4/80+ (resident) and CD11b+ (monocyte-derived) macrophages in the integrin b1 KO skin. F4/80+ tissue-resident macrophages have been shown to home to the skin as early as E13.5 (Hoeffel et al., 2012). We found a significant increase in the number of F4/80+ cells recruited at the DEJ of integrin b1 KO skin compared to the WT littermates by immunofluorescence (Figure 2A), corroborated with a 2-fold increase by flow cytometry (Figure 2B). We found that the number of CD11b+ macrophages were significantly higher in the integrin b1 KO skin as early as E16.5 (Figure 2C), where they formed the predominant immune infiltrate. Flow cytometric analyses confirmed a 3-fold increase in the CD11b+ populations of E18.5 integrin b1 KO skin (Figure 2D). Interestingly, the recruitment of myeloid-monocytic cells was observed to be followed by leukocytic infiltration around E17.5.
The CD3+ immune cells that traffic to sites of degradation include CD4+ and CD8+ T cells as well as gd T cells (Figures S2A–S2I). Here also, there was a significant increase in CD3+ cells by flow cytometry (Figure 2F). To further characterize the immune cell infiltrate, we looked at mast cells, known to be present in skin and mucosal tissue and responsible for allergic inflammation characterized by high IgE secretion (Amin, 2012). Incidentally, we do not observe any changes in mast cell numbers (Figures S2J–S2L), thus ruling out allergen-mediated inflammation. Angiogenesis is often associated with inflammatory diseases, aiding increased recruitment of immune cells to the site of injury or infection (Vanicchi et al., 2015). However, we found that increased immune trafficking to the skin was not due to angiogenesis (Figures S2M and S2N).

It has been shown that inflammatory responses in skin can be attributed to loss of barrier integrity through aberrant terminal differentiation programs (Lessard et al., 2013). Interestingly, b1 KO mice develop a normal epidermal barrier as experimentally shown by toluidine blue staining (Figures 2G and 2H). Therefore, an integrin b1 KO embryonic model is characterized by early recruitment of proinflammatory cells, uniquely independent of external damage cues such as a barrier breach or defects in terminal differentiation. We hypothesized that a paracrine recruitment of immune populations could be the potential source for the proinflammatory cytokine production and MMP activity, thus propa-
gating a damage response and eliciting local matrix remodeling.

### The ECM Degradation Phenotype Is Exacerbated by Immune Cells Recruited to the DEJ and Can Be Ameliorated by the Use of Anti-inflammatory Drugs

To test our hypothesis that the inflammatory cascade produced by the myelodominant infiltrate was responsible for ECM remodeling, we treated integrin b1 KO embryos with antiinflammatory drugs. Specifically, pregnant ITGb1fl/fl mice (mated with ITGb1fl/+;K14-Cre males giving integrin b1 KO and WT littermates) were administered with the NSAIDs aspirin and celecoxib for 3 consecutive days (corresponding to embryonic stages E15.5–E17.5). NSAIDs target cyclooxygenases (COX), a class of proinflammatory enzymes responsible for high levels of prostaglandin production during inflammation and pyrogenic responses (Baron and Sandler, 2000; Dannenberg and Subbaramaiah, 2003). After NSAID treatment, WT and b1 KO embryos were recovered from drug-treated pregnant females at E18.5 and the impact on immune response and ECM degradation was assessed. There was no impact of NSAIDs or vehicle DMSO treatment on average litter size or health of mother or pups (data not shown). Analyses of dorsal skin tissue from drug-treated integrin b1 KO embryos revealed a significant reduction in total numbers of F4/80+ tissue-resident macrophages, CD11b+ monocyte-derived macrophages, and CD3+ T cells along with a marked reduction in MMP activity at the DEJ, compared to vehicle-treated embryos (Figures 3A–3L). This was corroborated with analyses of the immune compartment by flow cytometry, as evidenced by an overall decrease in the CD3+ T lymphocyte and macrophage populations upon NSAID treatment (Figure 3P). The reduction in dermal immune infiltrate in NSAID-treated embryos was associated with a significant amelioration of fragmented ECM (Figures 3M–3O). Remarkably, the normalization of ECM in the drug-treated embryos also resulted in de novo hair follicle morphogenesis, which is otherwise developmentally attenuated in the integrin b1 KO skin (Figures 3N and 3O). This suggested that the disruption of the BM by the immune cells preceded the formation of hair placodes. We verified that there was no impact of NSAID treatment on BM structure or immune infiltration in treated WT littermates (Figure S3). We quantified the extent of phenotypic rescue by measuring the

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**Table 1. List of Genes Upregulated in NGS**

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<tr>
<th>Cytokines (Gene ID)</th>
<th>Fold Change (p value &lt; 0.05)</th>
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<tr>
<td>IL10</td>
<td>7.659</td>
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<tr>
<td>IL23x</td>
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<td>IL12x</td>
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<td>CCL17</td>
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<td>CCL2</td>
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<td>CCL21b</td>
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<td>MMP19</td>
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(Figures 2E and 2F). The CD3+ immune cells that traffic to sites of degradation include CD4+ and CD8+ T cells as well as γδ T cells (Figures S2A–S2I). Here also, there was a significant increase in CD3+ cells by flow cytometry (Figure 2F). To further characterize the immune cell infiltrate, we looked at mast cells, known to be present in skin and mucosal tissue and responsible for allergic inflammation characterized by high IgE secretion (Amin, 2012).
disrupted distribution of ECM components by immunofluorescence. There was a significant reduction in BM disruption in NSAID-treated β1 KO embryos, when compared to vehicle-treated controls (Figure 3R). qRT-PCR analysis of skin from β1 KO embryos treated with NSAIDs showed marked downregulation of inflammation-associated genes as well as MMPs associated with matrix remodeling (Figures 3Q, compare to 1H). Based on the phenotypic rescue of inflammation-associated ECM degradation by NSAIDs, we concluded that proinflammatory prostaglandin-dependent cascades are likely mediators of this embryonic remodeling. Notably, we show here that treating pregnant mice with NSAIDs can rescue ECM disruption phenotype, MMP activity, and concomitant immune infiltration in developing skin of in utero integrin β1 KO embryos.

The Strong Dermal Inflammatory Response in Integrin β1 KO Embryos Is Driven via TGF-β1-TNC Pathway in an Integrin β6-Dependent Manner

We have previously shown that conditional loss of integrin β1 results in a compensatory increase of integrin β6 in the epidermal compartment (Bandyopadhyay et al., 2012; RagHAVAN et al., 2003). In integrin β1 KO animals that display mosaic expression of integrin β1 due to Cre inefficiency (MOS), we found integrin β6 expression to be specifically upregulated in regions lacking integrin β1 (Figure 4A), corroborating our previous reports. Notably, we observed the highest amount of dermal immune infiltration in regions lacking epidermal integrin β1 and instead expressing integrin β6 (Figure 4B). Based on these data, we hypothesized that increased epidermal expression of integrin β6 upon conditional loss of integrin β1 triggers proinflammatory cascades and associated matrix remodeling. The relationship between integrin β6 and TGF-β1 is well established in an inflammatory context (Henderson and Sheppard, 2013; Munger et al., 1999) and provided us with the rationale to test the role of TGF-β1 signaling in potentiating the observed immune response. Indeed, integrin β6 has been shown to bind to the latency associated peptide (LAP) of TGF-β1 through its RGD motif, causing the release of free TGF-β1 (Morris et al., 2003; Munger et al., 1999; Yu and Stamenkovic, 2000). One of the direct targets of TGF-β1 signaling in dermal fibroblasts...
is TNC (Jinnin et al., 2004). We analyzed E16.5 embryonic skin to elucidate the earliest molecular events associated with the observable recruitment of immune cells and found regions of high integrin β6 expression to co-localize with regions expressing TNC (Figures S4A and S4B). This was associated with increased TGF-β1 in E16.5 integrin β1 KO skin compared to WT (Figures S4C and S4D). We assayed for nuclear pSMAD2/3 as a marker of active TGF-β1 signaling (Schmierer and Hill, 2007) and observed abundant nuclear expression in integrin β1 KO dermis as compared to WT, where it is mostly localized in the developing hair placodes (Figures S4E and S4F).

To directly test the role of integrin β6 in activating TGF-β1 in β1 KO skin, we blocked the function of integrin αvβ6 in utero by using the functional blocking antibody 10D5 (Huang et al., 1998). To target the antibody to the developing epidermis, we encapsulated it in self-assembled liposomes consisting of cationic lipid and DOPC (Figure S6). The liposomes containing 10D5 or the IgG control were injected in utero into E14.5 β1 KO or WT embryos and subsequently recovered E18.5 embryos for analysis.

Figure 3. Treating β1 KO’s with NSAIDs Reduces Inflammatory Response and MMP Activity and Rescues the ECM Phenotype

(A–C) Expression of tissue-resident macrophage marker F4/80 (green) and Laminin 5 (red) in DMSO-, aspirin-, and celecoxib-treated KO embryos.

(D–F) Expression of monocyte-derived macrophage marker CD11b (green) and Laminin 5 (red) in DMSO-, aspirin-, and celecoxib-treated KO embryos.

(G–I) Expression of pan-T cell marker CD3 (green) and Laminin 5 (red) in DMSO-, aspirin-, and celecoxib-treated KO embryos.

(J–L) In situ zymography of DMSO-, aspirin-, and celecoxib-treated KO embryos.

(M–O) Tiled images of the expression of β1 (green) and Laminin 5 (red) in DMSO-, aspirin-, and celecoxib-treated embryos. Note that the expression of Laminin 5 is significantly less spread in aspirin and celecoxib-treated embryos.

(P) FACS analysis of the total number of F4/80+, CD11b+, and CD3+ cells from whole skin samples taken at E18.5 from animals treated with DMSO, aspirin, or celecoxib (* indicates p < 0.05 and ** indicates p < 0.01; n = 3) (the error bars indicate SD, N.D. denotes not detected).

(Q) qRT-PCR for cytokines, chemokines, and MMPs performed on RNA isolated from E18.5 whole skin of animals treated with DMSO, aspirin, or celecoxib. The fold change values of WT are normalized to 1 (the error bars indicate mean ± SEM).

(R) Quantification of BM disruption N=atleast 10 field; error bars indicate +/– SD. The dotted lines indicate the upper most layer of skin in (A), (I), and (M)–(O) and DEJ in (J)–(L), epidermis (Epi), dermis (De). Arrowheads indicate leukocytes in A–L, nascent hair follicles in M–O. The scale bars represent 10 μm.

See also Figure S3.
10DS-injected embryos showed lower expression of TNC, extracellular TGF-β1, and pSMAD2/3 compared to the IgG-treated KO embryos (Figures S4J, S4K, S4N, S4O, S4R, and S4S), suggesting that integrin β6 is a potent and direct activator of TGF-β1 in β1 KO animals. Interestingly, the ECM organization was also rescued to some extent in the 10DS-treated embryos compared to IgG control (Figures S4V and S4W). WT animals treated with 10DS displayed lower levels of pSMAD2/3 in developing placodes compared to the IgG-treated controls (Figures S4P and S4Q) and generally displayed smaller hair placodes (Figures S4I, S4M, and S4U) compared to the control (Figures S4H, S4L, and S4T).

Several components of the TGF-β1 signaling pathway were distinctly upregulated in the dermis of integrin β1 KO skin, including TNC (Figures 4E–4H). TNC acts as a ligand for TLR4 to mediate inflammatory response via nuclear translocation of the p65 subunit of NF-κB, leading to secretion of proinflammatory chemokines (Midwood et al., 2009). We observed a distinct

Figure 4. Role of the Integrin β6-TGFβ1-TNC Axis in Setting up the Inflammatory Response
(A) Expression of β1 (red), integrin β6 (green), and Laminin 5 (white) in back skin of E18.5 MOS. Note the reciprocity of the expression of β1 and integrin β6.
(B) Expression of F4/80 (green), integrin β6 (red), and Laminin 5 (white) in back skin of E18.5 MOS. Note that the immune infiltrate is associated with expression of integrin β6 and loss of ECM organization.
(C and D) Expression of integrin β6 (green) and TNC (red) in back skin of E18.5 WT and β1 KO embryos.
(E and F) Expression of TGFβ (green) in E18.5 WT and β1 KO embryos.
(G and H) Expression of TGFβR1 (red) and K5 (green) in E18.5 WT and β1 KO embryos.
(I and J) Expression of TNC (red) and TLR4 (green) in E18.5 WT and β1 KO embryos.
(K and L) Expression of K5 (white), F4/80 (green), and Myd88 (red) in E18.5 WT and β1 KO embryos. Note that the Myd88 expression is seen in F4/80 positive cells and in some basal cells.
(M and N) Expression of p65NF-κB (red) and K5 (green) in E18.5 WT and β1 KO embryos.
(O and P) Expression of K5 (green) and Cox2 (red) in E18.5 WT and β1 KO embryos. The dotted lines indicate the upper most layer of skin in all images except (E) and (F), where it indicates DEJ. epidermis (Epi), dermis (De). The scale bar represents 10 μm.
(Q) RT-qPCR validation of β6-TGFβ1-TNC pathway. The data are average of two independent replicates (the error bars indicate mean ± SD). The WT fold change values are normalized to 1.
(R) Immunoblot analysis with quantification of protein levels from E18.5 WT and KO whole skin.
(S) Model of β6-TGFβ1-TNC axis in β1 KO embryos shows: (1) the upregulation of integrin β6 in epidermis of β1 KO embryos; (2) integrin β6 binds to TGFβ1-LAP, (3) TGFβ1 is released from its LAP upon binding to integrin β6 and binds a TGFβ1R1/7TGFR1 receptor dimer on extracellular binding domains, (4) TNC is localized at the DEJ, (5) TNC binds TLR4/Myd88 in the dermis (primarily in the macrophages), (6) Proinflammatory cytokines are released by nuclear NF-κB driven transcription resulting in, (7) the recruitment of additional tissue-resident and monocyte-derived macrophages, and (8) the release of active MMPs at the DEJ resulting in remodeling of the BM.
See also Figures S4 and S6.
TNC enrichment in the dermis of symptomatic integrin β1 KO skin (Figures 4C and 4D), which was also corroborated by qRT-PCR and protein expression (Figures 4Q and 4R). Downstream TLR4-dependent NF-κB signaling was found to be active in integrin β1 KO dermis (Figures 4I–4N and 4R). qRT-PCR performed on E16.5 integrin β1 KO skin showed enhanced expression of key players in this molecular pathway (Figure S4-G), thus corroborating active TLR signaling. Lastly, we verified that Cox2 expression was in fact higher in KO embryos, indicating the TLR-mediated inflammatory cascade results in Cox2 expression in a NF-κB-dependent manner (Figures 4O–4R). We thus provide substantial evidence that the observed inflammatory cascade in integrin β1 skin is triggered by expression of integrin β6 as early as E16.5. Our model is that integrin β6 activates paracrine TGF-β1 signaling in the vicinity of the BM, resulting in increased expression of TNC by the underlying fibroblasts. TNC binds TLR4/Myd88 (Midwood et al., 2009) predominantly in the dermal compartment leading to nuclear NF-κB signaling. We hypothesize that NF-κB signaling in dermis leads to recruitment of tissue-resident and circulating macrophages as well as effector T lymphocytes to the DEJ, resulting in MMP activation and remodeling of the BM of integrin β1 KO skin (Figure 4S).

**In Utero Treatment of Integrin β1 KO Embryos with TGF-βR1 Inhibitor Leads to Amelioration of ECM Disruption and Immune Infiltration**

In order to directly address the role of the TGF-β1 pathway in setting up the observed ECM phenotype and associated immune infiltration, we decided to target the pathway using a small molecule inhibitor SD208 that specifically targets TGF-βR1 (Uhl et al., 2004). We proceeded to modulate the availability of TGF-β1 by injecting liposome-encapsulated SD208 (5 μg) into pregnant female mice in utero. Remarkably, we observed a significant rescue of ECM degradation upon administration of SD208, when compared to control liposome-treated integrin β1 KO embryos (Figures 5I, 5J, S5, SSD, and SSL). This was associated with a clear reduction in immune infiltration by T lymphocytes and macrophages (Figures 5A–5F, S5N, S5P, S5R, ST, SY, and S5X). The expression of TNC and integrin β6, which are direct targets of TGF-β1, are also greatly diminished upon administering SD208 (Figures 5K, 5L, SSF, and SSH), indicating efficacy of the treatment. As SD208 targets SMAD activation (Uhl et al., 2004), we assayed for activated pSMAD2/3 and found it to be reduced in the dermis of SD208-treated β1 KO embryos (Figures 5M, 5N, S5J, and SSL). There was much lower pSMAD2/3 expression in WT embryos treated with SD208 when compared to vehicle, especially in the interfollicular epidemis (Figures S5I and S5K). Interestingly, we also found fewer hair placodes developing in the WT back skin, which was expected since the TGF-β signaling pathway is important for hair morphogenesis (Klopcic et al., 2007). Additionally, this served as a control for the efficacy of TGF-β blocking by SD208. We also observed a strong reduction in Myd88 and NF-κB levels (Figures 5O–5R) and a marked reduction in MMP activity by in situ zymography (Figures 5G and 5H). The expression of collagen IV, which was disrupted in the β1 KO, was restored in the DEJ of SD-208-treated β1 KO animals (Figures 5S and 5T). Taken together, these data provide strong direct evidence for the role of paracrine the TGF-β1 pathway in setting up the earliest events in the sterile inflammatory response observed in integrin β1 KO embryos.

**In Utero Treatment of Integrin β1 KO Embryos with NF-κB Inhibitor Leads to Amelioration of ECM Disruption and Immune Infiltration**

Presence of TNC and activation of TLR4/Myd88 in β1 KO dermis (Figures 4I–4N) is in line with previous reports of TNC activity as an endogenous activator of TLR signaling (Midwood et al., 2009) and provided us with a rationale to test the necessity of NF-κB signaling in precipitating this dermal immune response. We pharmacologically manipulated NF-κB signaling using a well-known small molecule inhibitor Bay-11–7082, which efficiently abrogates the DNA-binding ability of NF-κB (Mori et al., 2002). Liposomes containing Bay-11 (2.5 μg) or control liposomes were injected in utero into E14.5 β1 KO and WT embryos and subsequently recovered at E18.5 for analysis. There were no significant differences observed in numbers of immune cells, MMP activity, ECM organization, or size of hair placodes in the Bay-11-treated WT animals compared to the control-treated animals (Figures 6A–6R). However, we observed a significant rescue of the ECM degradation in the Bay-11-treated β1 KO embryos (Figures 6S and 6T), associated with a decrease in T lymphocytes and macrophages (Figures 6C–6L). MMP activity was also markedly reduced in the Bay-11-treated embryos (Figures 6G and 6H). Taken together, these data provide strong evidence for the role of NF-κB in the inflammatory response seen in the KO embryos.

Altogether, we propose the following model for how an embryonic inflammatory response may be capable of remodeling the developing epithelial BM (Figure 7). In developing WT skin, normal organization of the BM is maintained by the expression of integrin β1. The normal prenatal immune contexture primarily involves a modest pool of resident macrophages and naive T cells, which form the early surveillance response. The loss of epithelial integrin β1 is associated with severe BM disruptions characterized by an increased recruitment of monocytes/macrophages and T cells and an increased activity of matrix remodeling enzymes. Our data show that in the absence of integrin β1, there is an upregulation of integrin αvβ6 in basal keratinocytes, which in turn induces proinflammatory cytokine production in the dermis. By treating the embryos with NSAIDs, we show that this embryonic inflammatory cascade is the primary reason behind BM remodeling. Paracrine TGF-β signaling at DEJ leads to TNC-dependent activation of NF-κB in dermis, which results in a “stress response” that drives prostaglandin production. Such a danger signal drives infiltration of leukocytes into the DEJ, promotes remodeling through secreted MMP activity, and ultimately aggravates BM disruption.

**DISCUSSION**

Our study reveals somewhat unexpected insights into the mechanisms by which integrin β1 maintains epidermal homeostasis and tissue integrity during development. The integrin β1 KO model provides a unique platform to study the earliest events involved in damage-associated sterile inflammation, the study of which could have major implications in the treatment of skin...
blistering disease and skin cancers. Specifically, this study establishes a direct link between the recruitment of phagocytic cells and MMP-dependent remodeling of the BM during embryonic skin development. In doing so, we can reconcile the exaggerated ECM-degradation phenotype with a non-mechanosensory role for integrin $\beta_1$ at the basal epidermis.

Although inflammatory responses in embryonic skin have not been well characterized previously, there are published reports of immune cell infiltration in embryonic skin of Ets1 transgenic mice (Nagarajan et al., 2010) and in Involutin, Envolplakin, and Periplakin triple KO mice (Sevilla et al., 2007). However, both these models display a loss-of-barrier phenotype and hence are vulnerable to extrinsic insults and ensuing immune response. In addition to embryonic models, the adult $\beta_1$ KO mouse (Brakebusch et al., 2000) also displayed defective hair growth, aberrant ECM production, and infiltration of F4/80+ macrophages and T lymphocytes. It was suggested that the recruitment of immune cells in the KO animals could be in response to malformed hair follicles, but no direct correlation was made with ECM disorganization. To the best of our knowledge, this robust prenatal innate immune response elicited by local damage-induced chemokine cues, and set up in the presence of normal terminal differentiation as well as an intact skin barrier, has not been previously reported.

In this study, we demonstrate that the exaggerated ECM degradation phenotype observed in integrin $\beta_1$ KO embryos is the result of an unexpected embryonic inflammatory response. This inflammatory response is triggered by the loss of integrin $\beta_1$ in epidermal cells and associated with concomitant upregulation of integrin $\beta_6$. The role of integrin $\beta_6$ in abnormal wound healing...
and TGF-β mediated fibrosis (Häkkinen et al., 2004) as well as immunosuppression (Bandyopadhyay and Raghavan, 2009) is well documented. In this study, we have further dissected the lesser-known function of integrin αvβ6 in driving inflammation in a non-cell autonomous manner via TGF-β1 signaling. Specifically, we show that increased expression of integrin β6 in β1-deficient cells activates TGF-β1 signaling, resulting in the up-regulation of TNC expression. TNC serves not only as a ligand for integrin β6, but also as a potent DAMP (Goh et al., 2010). The TNC-TLR4-Myd88 axis in innate immune cells results in NF-κB driven cytokine and chemokine production in the dermis. This, in turn, leads to recruitment of monocytes/macrophages and T lymphocytes, subsequent MMP activity, and an exaggerated disruption of the ECM. Importantly, treatment of integrin β1KO embryos with either antiinflammatory drugs (NSAIDs) or with small molecular inhibitors of TGF-βR1 and NF-κB resulted in a marked reversal of ECM defects, suggesting an unexpected role for integrin β1 in epidermal immune homeostasis by regulating activity/expression of integrin β6 and TGF-β1 effectors.

The role of inflammatory immune cells in ECM remodeling is well known in the context of diseases like rheumatoid arthritis (RA), osteoarthritis (OA), and several cancers (Lu et al., 2011). There is mounting evidence that suggests that individuals with chronic inflammatory diseases such as pancreatitis and Crohn’s disease are predisposed to developing certain cancers (Cousens and Werb, 2002). This has been attributed to the persistence of proinflammatory stromal cells that can potentiate and shelter neoplastic lesions. Tumor-associated macrophages (TAMs) and immature granulocytes have been shown to produce excessive protumorigenic cytokines as well as remodeling enzyme MMP9, resulting in disruption of the ECM as well as enhanced growth factor processing (Chanmee et al., 2014; Riabov et al., 2014). The resultant angiogenic spurt, increased proliferation, and enhanced invasion contribute to aggressive disease, thus underpinning the protumorigenic role of inflammatory ECM remodeling. It hence follows that drugs aimed at limiting proinflammatory cascades can be promising as treatment modalities. Indeed, several large-scale retrospective studies suggest that cancer patients on long-term NSAID therapy are protected from metastatic events (Algra and Rothwell, 2012; Rothwell et al., 2010). The in vivo NSAID experiments described in our report successfully demonstrate that ECM degradation in the β1 KO model is driven by classical prostanoid-dependent inflammatory cascades. These experiments

Figure 6. In Utero Injection of Bay-11 in β1 KO Embryos Rescues the ECM Phenotype

(A–D) Expression of tissue-resident macrophage marker F4/80 (green) and Laminin 5 (red) in vehicle-treated and Bay-11-treated E18.5 WT and β1 KO embryos.

(E–H) Expression of monocyte-derived macrophage marker CD11b (green) and Laminin 5 (red) in vehicle-treated and Bay-11-treated E18.5 WT and β1 KO embryos.

(I–L) Expression of pan-T cell marker CD3 (green) and Laminin 5 (red) in vehicle-treated and Bay-11-treated E18.5 WT and β1 KO embryos.

(M–P) In situ gelatin zymography in vehicle-treated and Bay-11-treated E18.5 WT and β1 KO embryos.

(Q–T) Expression of β1 (green) and Laminin 5 (red) in vehicle-treated and Bay-11-treated E18.5 WT and β1 KO embryos. The dotted lines indicate the upper most layer of skin in all images except (M–P), where they indicate DEJ. epidermis (Epi), dermis (De). The scale bar represents 10 μm.

See also Figure S6.
also provide proof-of-concept for this unique mode of drug administration that warrants further study.

Taken together, our work suggests a key role played by integrin β1 in regulating tissue homeostasis and in maintaining an immuno-protective microenvironment during tumultuous remodeling of the ECM during embryonic development. The integrin β1 KO model also serves as a fantastic model to study early events in sterile inflammatory diseases.

**EXPERIMENTAL PROCEDURES**

**Animals**

Generation of the β1 KO animals has been described elsewhere (Raghavan et al., 2000). Animals of mixed background (C57BL6/CD1) were used for all experiments. ITGβ1fl/fl females and ITGβ1fl/+; K14-Cre male mice were crossed to WT females at E11.5. Embryos were collected at E16.5 and E18.5. Whole-mount barrier assay was performed as described previously (Byrne et al., 2016). Animals of mixed background (C57BL6/CD1) were used for all experiments.

**Flow Cytometry**

E18.5 embryonic skin was treated with dispase (1 U/ml) for 1 hr at 37°C to separate the epidermis from dermis. The epidermis and dermis were then treated with 0.025% trypsin and collagenase (0.2 mg/ml), respectively, to separate the epidermis from dermis. The epidermis and dermis were then treated with 0.025% trypsin and collagenase (0.2 mg/ml), respectively, to separate the epidermis from dermis.

**Immunofluorescence**

10 μm cryosections of embryos were fixed for 10 min at RT in 4% paraformaldehyde or at –20°C in 100% ice-cold acetone. This was followed by washes with 1× PBS and permeabilization with PBST (0.2% Triton X-100 in PBS; or as mentioned in antibody datasheet). The sections were blocked with 5% normal donkey serum in 1× PBS and incubated with primary antibodies for 40 min at RT and mounted using Mowiol mounting medium. The primary antibodies used were: anti-integrin β1, TNC, fibronectin, HSPG, collagen IV, and entactin (Millipore); anti-CD3, F4/80, γ/δTCR, CD4, and CD8 (eBiosciences); anti-TLR4, Myd88, Cox2, and pSMAD2/3 (Abcam); NF-κB, GAPDH (Cell Signaling); anti-TGF-β1 (R&D Systems); antilaminin 5 (332) (gift from Bob Burgess); anti-K5 (gift from Colin Jamora); antiintegrin β6 (gift from Sheila Violette, Biogen Idec, Boston), and anti-K6 (gift from Satrajit Sinha). Images were acquired on a Zeiss LSM780 or LSM780 confocal microscope.

**Toluidine Blue Staining for Mast Cells**

The frozen sections were fixed in 4% paraformaldehyde. Toluidine blue stock solution was made by dissolving 1 g of toluidine blue in 100 ml of 70% ethanol. The sections were incubated for 3 min with toluidine blue stain working solution (pH 2.3), washed three times with distilled water, and mounted with DPX mountant (Sigma-Aldrich).

**Toluidine Blue Dye Penetration Assay**

Whole-mount barrier assay was performed as described previously (Byrne et al., 2016). Briefly, E16.5 embryos were treated in successional methanol gradients (25%, 50%, 75%, 100%, 75%, 50%, and 25%), equilibrated with PBS dipped in 1% toluidine blue dye for 30 s–1 min and then destained in PBS until pattern appeared. Embryos were embedded in 1% agar and photographed using a dissection microscope at 4× magnification.

**In Situ Zymography**

Localized gelatinolytic activity of the MMP in unfixed cryosections of embryos were determined by using DG gelatin (Invitrogen) as a substrate. Frozen slides were thawed for 5 min and washed with PBS twice for 5 min each to remove OCT. This was followed by preincubation of slices in MMP activation buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM CaCl2, and 0.2 mM sodium azide) or 20 mM EDTA (control) in an incubation chamber at 37°C. Slides were then incubated for 2 hr at 37°C with DG gelatin (1 mg/ml diluted 1:50 in activation buffer), followed by three washes with PBS. Slides were then fixed with 4% paraformaldehyde for 10 min and stained with DAPI and mounted in Mowiol mounting medium (Sigma-Aldrich). Slides were imaged using a Zeiss LSM780 or LSM 780 confocal microscope.

**RNA Extraction and Real-Time qPCR**

Total RNA was extracted from whole skin of WT and KO E16.5 and E18.5 embryos using TRizol reagent (Invitrogen) according to manufacturer’s instructions. RNA was then quantified using a Nanodrop spectrophotometer (Thermo Scientific). Reverse transcription was performed using GoScript Reverse Transcriptase (Promega). The copy number of GAPDH served as an internal control for gene expression analysis.
instructions. Total RNA was dissolved in nuclease-free water and quantified using Nanodrop (Thermo Scientific). 1 μg of total RNA was used to synthesize cDNA using SuperScript III RT First Strand cDNA Synthesis Kit (Invitrogen). Real-time PCRs were performed on an ABI/7500HT system. mRNA expression level was quantified by using the ΔΔCT method and the gene expression was normalized to GAPDH. The list of primers used is provided in Table S1.

Western Blotting
E18.5 whole skin tissue from WT and KO embryos was snap-frozen in liquid nitrogen and pulverized using a tissue smasher. Tissue was resuspended in RIPA lysis buffer (Sigma-Aldrich) with protease inhibitor cocktail (Roche) and incubated on ice for 30 min. The lysates were sonicated and centrifuged at high speed for 15 min. The protein was estimated using a BCA kit (Promega). 50 μg of total protein per lane was electrophoresed in sodium dodecyl sulfate polyacrylamide gels (8%-10% SDS-PAGE) and transferred onto PVDF membranes (Bio-Rad). The blots were blocked using 5% BSA and incubated with primary antibody overnight at 4°C. The blots were washed with 1× TBST and incubated with HRP conjugated secondary antibodies for 1 hr at RT. The blots were developed using chemiluminescence on ImageQuant LAS 4000 mini biomolecular imager.

Anti-inflammatory Drug Treatment
ITGβ1+/− females and ITGβ1+/−;K14-Cre males were crossed as described below. Pregnant females were started on the drug regimen when the embryos were E15.5 from date of plug. Celecoxib (10 mg/kg) (Selectech Chemicals) and aspirin (10 mg/kg) (Sigma-Aldrich) were dissolved in a vehicle of sunflower seed oil (Cat 88921, Sigma-Aldrich) with 5% (v/v) DMSO (American Bioanalytical). For 3 consecutive days (E15.5, E16.5, and E17.5), the mice were gavaged by oral gavage using 1 ml gavage needle. Control animals were administered vehicle alone at similar volumes for the same duration. For each of the treatments, at least ten females were treated with celecoxib, aspirin, or vehicle control. Embryos were recovered at E18.5 and either embedded in OCT or the epidermis and dermis were separated and stored in RNA later (Fisher) for RNA isolation and qRT-PCR analysis.

In Utero Injections of TGF-β1 Inhibitor SD-208, NF-κB Inhibitor Bay 11-7082, and Integrin αvβ6 Blocking Antibody 10D5
0.5 mg of cationic lipid with cyclic (N,N'-dimyristyl cyclic 3,4-dihydroxy-pyrrolidinium) head group, 1 mg of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) (Avanti polar), and 250 μg of SD208/Bay-11-7082 (Sigma-Aldrich) was dissolved in 80 μl of ethanol and 10% of Vitamin E TPGS (Sigma-Aldrich) at 80°C in a glass vial. Milli-Q water was preheated to 80°C. Water was slowly added into glass vial containing lipid mixture under constant stirring condition and continued vortex for 2–3 min at RT to produce multilamellar vesicles (MLVs). MLVs were then sonicated in an ice bath until clarity using a Qsonica Sonifier at 100% duty cycle and 5J output power to produce small unilamellar vesicles. MLVs were then sonicated in an ice bath until clarity using a Qsonica Sonifier at 100% duty cycle and 5J output power to produce multilamellar vesicles. MLVs were then sonicated in an ice bath until clarity using a Qsonica Sonifier at 100% duty cycle and 5J output power to produce small unilamellar vesicles (SUlV). The integrin αvβ6 blocking antibody 10D5 was encapsulated in the following manner. To make 3.6 mM liposome, 1,212 mg of cyclic lipids and 2,826 mg of DOPC were dissolved in chloroform (500 μl) in a glass vial. The solvent was removed with a thin flow of moisture-free nitrogen gas and the lipid film was kept for drying under high vacuum for 6 hr. 1 ml of 5% glucose solution was added to the vacuum dried lipid films and the mixtures were allowed to swell overnight. The vials were then vortexed for 2–3 min at RT to produce MLVs. MLVs were then sonicated in an ice bath until clarity using a Qsonica Sonifier at 100% duty cycle and 5J output power to produce small SUVs. 10 μl of liposome was added to 1 μl (2 mg/ml) of antibody integrin αvβ6 blocking antibody 10D5 (gift from Dean Sheppard, UCSF) for in utero injection. The amniotic fluid of embryos (E15.5) was injected either with the drug or the vehicle control on the ventral side, where the placenta attaches. This allowed the drug to be evenly distributed and absorbed by the skin before the establishment of a terminal barrier. We recovered the injected embryos between E18–E18.5 and analyzed skin phenotype as described elsewhere.

Statistical Analysis
Mean, SD, SEM, and statistical significance were calculated using GraphPad v6.0e (GraphPad Software). Where appropriate, two-tailed unpaired t test or Mann-Whitney U test was used for experiments involving only two groups based on variance of data set, and two-way ANOVA was used for comparison across multiple groups.

ACCESSION NUMBERS
The accession number for the NGS data reported in this paper is SRA: SRP078330.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.062.

AUTHOR CONTRIBUTIONS
S.R. conceived and supervised the study. A.S.K. performed all of the experiments. S.H. performed initial mouse experiments, qRT-PCR experiments, immunostaining, and image analyses. O.B. conducted the qRT-PCR experiments, helped with immunostaining, and assisted with mouse experiments. S.M. and P.K.V. designed and developed delivery formulations, provided SD-208, Bay-11 and 10DS formulations, assisted with the experiments, and helped in writing the methods. S.R., A.S.K., and S.H. analyzed the data and wrote the manuscript.

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