Functional Beta2-Integrins Restrict Skin Inflammation *In Vivo*

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Beta2-integrins and the important integrin regulator kindlin-3 are essential for leukocyte trafficking, but the role of beta2-integrins in regulating inflammation is still incompletely understood. Here, we have investigated skin inflammation in a mouse model where the kindlin-3 binding site in the beta2-integrin has been mutated (TTT/AAA-beta2-integrin knock-in), leading to expressed but dysfunctional integrins. We show that, surprisingly, neutrophil trafficking into the inflamed skin in a contact hypersensitivity model is normal in these mice, although trafficking of T cells and eosinophils into the skin is reduced. Instead, expression of dysfunctional integrins leads to increased mast cell and dendritic cell numbers in the skin, increased inflammatory cytokine production in the inflamed skin *in vivo*, and in mast cells *in vitro*. Furthermore, expression of dysfunctional integrins leads to increased dendritic cell activation and migration to lymph nodes and increased Th1 responses *in vivo*. Therefore, the kindlin-3/integrin interaction is important for trafficking of T cells and eosinophils but not absolutely required for neutrophil trafficking into the inflamed skin. Functional beta2-integrins also have a major role in restricting the immune response in the inflamed skin and lymph nodes *in vivo*, likely through effects on mast cell and dendritic cell numbers and activation.

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INTRODUCTION

Beta2-integrins (alphaLbeta2, LFA-1, alphaMbeta2, Mac-1, alphaXbeta2, and alphaDbeta2) have major roles in leukocyte trafficking and function, as shown by the rare genetic syndrome leukocyte adhesion deficiency type I (LAD-I). In this disorder, beta2-integrin expression is reduced or lost, leading to a deficiency in leukocyte trafficking into sites of infection. Patients with this disorder suffer from recurrent bacterial and fungal infections and display leukocytosis, periodontitis, and delayed wound healing (Etzioni, 2009).

Beta2-integrins require activation to mediate firm adhesion to the blood vessel endothelium under shear flow conditions. Integrins are regulated through the binding of talin and kindlin-3 to the beta-subunit cytoplasmic domain. Kindlin-3 is mutated in LAD-III, a genetic syndrome where integrin expression is normal but adhesion is dysregulated, leading to a similar immune deficiency as LAD-I, among other symptoms (Moser *et al.*, 2008; Malinin *et al.*, 2009; Moser *et al.*, 2009; Svensson *et al.*, 2009).

Both talin and kindlin-3 have been shown to be important for firm adhesion of neutrophils under shear flow conditions and work together to achieve the high-affinity conformation of the integrins (Lefort *et al.*, 2012). We have previously shown that the integrin/kindlin-3 interaction is required for T-cell adhesion under shear flow conditions and for trafficking of T cells into lymph nodes (Morrison *et al.*, 2013); whether the integrin binding property of kindlin is required for trafficking of neutrophils or other leukocyte subtypes is currently unknown.

In addition to their well-established role in leukocyte trafficking, beta2-integrins have recently been shown to mediate anti-inflammatory functions in the immune system. They have been shown to restrict toll-like receptor signaling in macrophages and dendritic cells (DCs; Bai *et al.*, 2012) and DC-mediated T-cell priming (Varga *et al.*, 2007; Balkow *et al.*, 2010). In addition, LAD-I patients have been described to have increased susceptibility to colitis (Uzel *et al.*, 2008) and periodontitis (Schmidt *et al.*, 2013). LAD-I periodontitis has recently been attributed to excessive IL-17 production (Moutsopoulos *et al.*, 2014). We have shown that the beta2-integrin/kindlin-3-cytoskeletal interactions restrict DC maturation, migration of DCs from the skin to the lymph nodes, and Th1 responses *in vivo* (Morrison *et al.*, 2014), further emphasizing the putative anti-inflammatory roles of beta2-integrins. Therefore, we were interested in investigating which role of beta2-integrins dominates in skin inflammation *in vivo*, leukocyte trafficking, or restriction of immune

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Abbreviations: DC, dendritic cell; KI, knock in; KO, knock out; LAD-1, leukocyte adhesion deficiency type I; LN, lymph node; OXA, oxazolone; WT, wild type

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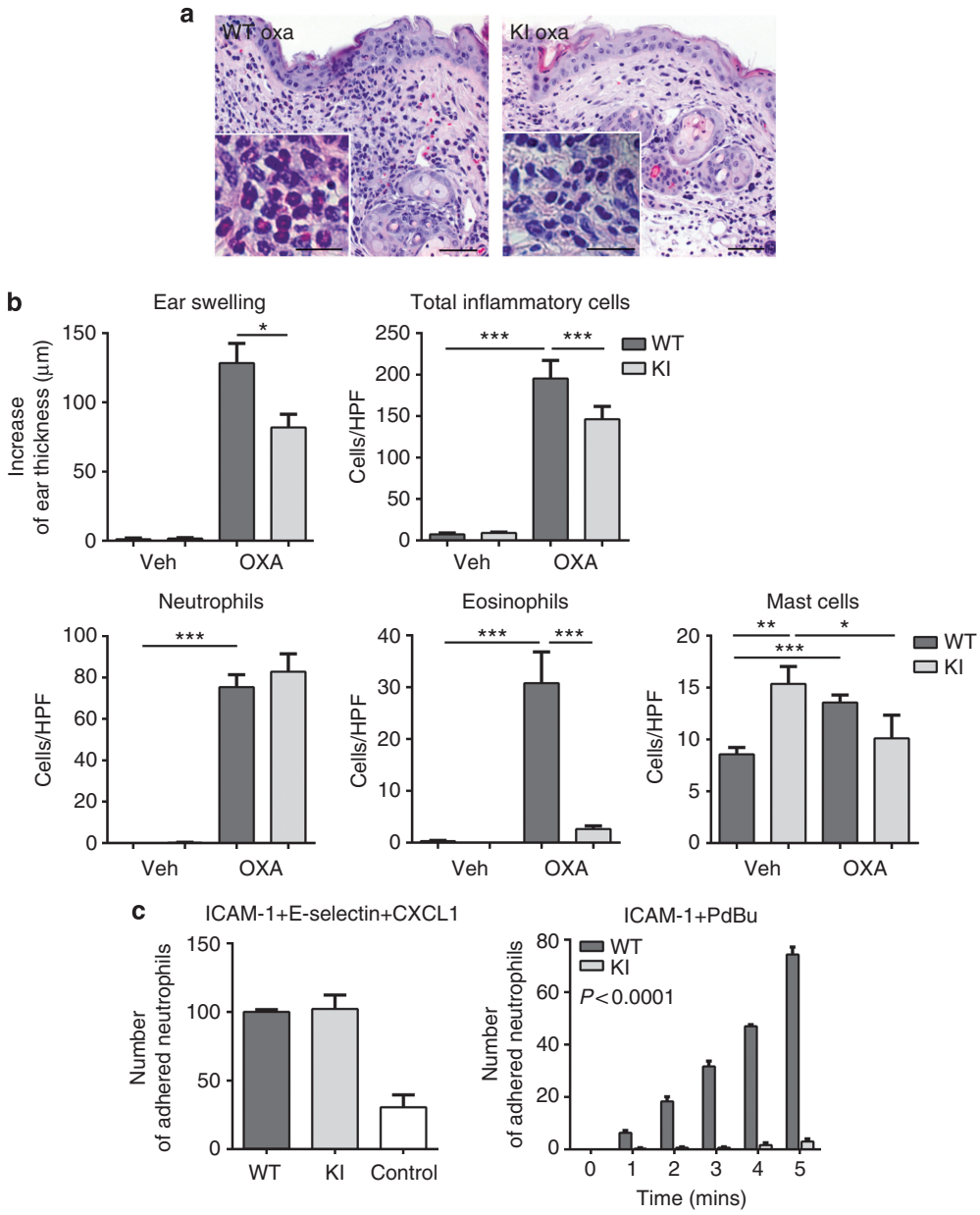

Figure 1. Neutrophil migration into inflamed sites is not dependent on the integrin/kindlin interaction. (**a**) Ear sections were stained with hematoxylin and eosin (H&E) after oxazolone (OXA) challenge. Inserts show eosinophils in wild-type (WT) skin and neutrophils in knock-in (KI) skin. Bar = 50 μm; Bar = 20 μm (inserts). (**b**) Total inflammatory cells and the number of neutrophils, eosinophils, and mast cells were counted from H&E, toluidine blue, and anti-mouse Ly-6G (neutrophils) stained ear sections, and thickness of the ear was measured in control and OXA-treated mice. Ear thickness was measured before OXA application and 24 hours after OXA treatment and the total increase is shown, $n = 8$. (**c**) Adhesion of isolated neutrophils from WT and KI mice was investigated under shear flow conditions at 5 dynes cm^{-2} on ICAM-1, CXCL-1, and E-selectin-coated plates, and controls were coated without ICAM-1. Adhered cells were counted after 5 minutes. Neutrophil adhesion was also investigated on ICAM-1-coated plates in the presence of PdBu at 0.3 dynes cm^{-2}. Adhered cells were counted after 1, 2, 3, 4, and 5 minutes, $n = 3$. Mean ± SEM is shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. HPF, high-power field; Veh, vehicle.

responses. To do this, we made use of a TTT/AAA–beta2–integrin knock-in (KI) mouse (Morrison *et al.*, 2013) in which the beta2-integrin/kindlin-3 interaction has been abolished.

Here, we show that, very surprisingly, and in contrast to the situation found in beta2-integrin (Grabbe *et al.*, 2002) and kindlin-3 (Moser *et al.*, 2009; Lefort *et al.*, 2012) knockout (KO) neutrophils, neutrophil adhesion under shear flow conditions, and neutrophil trafficking into the inflamed skin *in vivo,* is not absolutely dependent on the beta2-integrin/kindlin-3 interaction. However, beta2-integrin/kindlin links are important for trafficking of T cells and eosinophils into the inflamed skin. In addition, functional integrins (regulated by the beta2-integrin/kindlin-3 interaction) restrict skin mast cell and DC numbers, the production of inflammatory, Th1, Th2, and Th17 cytokines in the inflamed skin, and mast cell IL-4 production *in vitro*. Furthermore, this interaction restricts Th1 cytokine production in inflamed lymph nodes, likely by restricting DC migration from the inflamed skin into the lymph nodes.

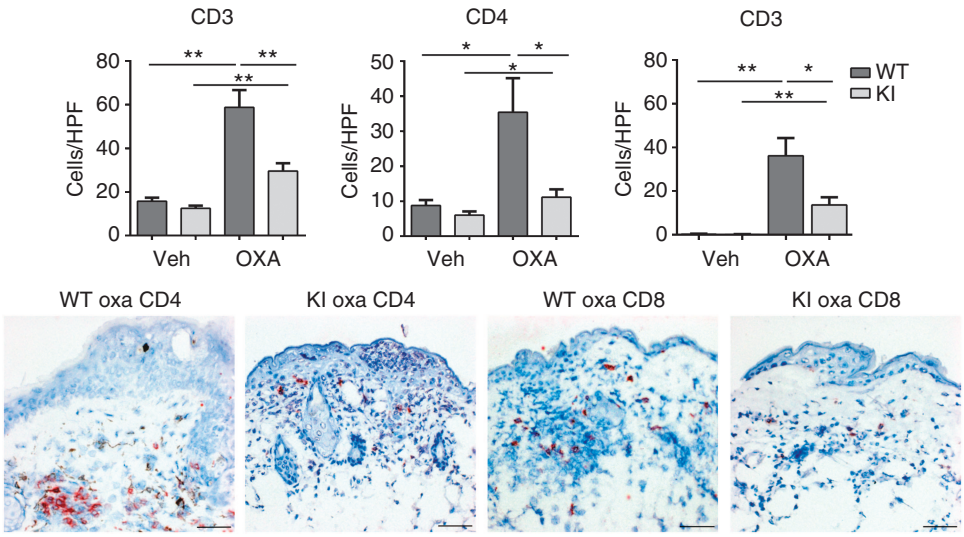

Figure 2. T-cell migration into inflamed sites is dependent on the integrin/kindlin interaction. Frozen ear tissue from oxazolone (OXA) challenged and control-treated wild-type (WT) and knock-in (KI) mice was stained with anti-CD3, anti-CD4, and anti-CD8 and counted manually, $n = 5–6$. Mean ± SEM is shown. $^*P < 0.05$; $^{**}P < 0.01$. Representative figures of WT and KI OXA–treated ear sections stained with anti-CD4 and anti-CD8 are shown.$^*P < 0.05$; $^{**}P < 0.01$. Bar = 50 μm. HPF, high-power field; Veh, vehicle.

Collectively, these results demonstrate that beta2-integrin/kindlin-3 connections have a role in leukocyte trafficking into the inflamed skin, but that these molecules also have a clear anti-inflammatory role in skin inflammation *in vivo*.

RESULTS

The kindlin-3/integrin interaction is not required for neutrophil trafficking into the inflamed skin

Beta2-integrins have previously been shown to be crucial for neutrophil and T-cell trafficking into the inflamed skin (Grabbe *et al.*, 2002), and kindlin-3 is important for neutrophil adhesion under shear flow conditions and for trafficking of neutrophils *in vivo* (Moser *et al.*, 2009; Lefort *et al.*, 2012). However, the role of the kindlin-3/beta2-integrin interaction in leukocyte trafficking into sites of inflammation is currently unknown. To investigate the role of the integrin/kindlin-3 interaction in leukocyte trafficking in skin inflammation, mice were sensitized with the chemical hapten oxazolone (OXA), challenged with OXA 5 days later, and inflammatory responses were determined. Surprisingly, and in contrast to beta2-integrin KO mice (Grabbe *et al.*, 2002), TTT/AAA-beta2-integrin KI mice displayed only a minor defect in mounting a hapten-specific response to OXA challenge (Figures 1a and b). In addition, neutrophil trafficking into the inflamed skin was normal in this model (Figure 1b and Supplementary Figure S1 online), although eosinophils were not found in the KI mice inflamed sites (Figure 1b and Supplementary Figure S2 online). The number of eosinophils in the blood, bone marrow, and spleen was normal in naive (unimmunized) KI mice, showing that the integrin/kindlin interaction does not regulate eosinophil development or proliferation (Supplementary Figure S3 online). In contrast, the number of mast cells was increased already in the uninflamed skin of KI mice, whereas the numbers of mast cells were similar in OXA-challenged KI and wild-type (WT) skin (Figure 1b). Degranulated mast cells were present in both groups.

As CXCL-1–induced neutrophil adhesion to ICAM-1/E-selectin under shear flow conditions has previously been shown to be dependent on kindlin-3 (Lefort *et al.*, 2012), we went on to investigate adhesion of TTT/AAA-beta2-integrin KI neutrophils *in vitro* to the beta2-integrin ligand, ICAM, with and without E-selectin. These studies revealed that, although KI neutrophil adhesion to ICAM-1 in the presence of phorbol ester was severely deficient, adhesion to ICAM-1/E-selectin in the presence of CXCL-1 was not affected in TTT/AAA-beta2-integrin KI neutrophils (Figure 1c). Together, these results imply that the interaction between kindlin-3 and the beta2-integrin is not strictly required for neutrophil adhesion under shear flow conditions or for neutrophil trafficking into sites of inflammation *in vivo*.

T-cell trafficking into the inflamed skin is reduced in TTT/AAA-beta2-integrin KI mice

The interaction between kindlin-3 and the beta2-integrin has been shown to be important for T-cell adhesion under shear flow conditions and for naive T-cell trafficking into lymph nodes (Morrison *et al.*, 2013), and beta2-integrins (Grabbe *et al.*, 2002) and kindlin-3 (Cohen *et al.*, 2013) have been shown to be important for the trafficking of T cells into the inflamed skin. We show here that TTT/AAA-beta2-integrin KI mice had reduced numbers of $CD8^+$ and $CD4^+$ T cells in the OXA-challenged ear tissue, confirming that both $CD4^+$ and $CD8^+$ T-cell trafficking into the inflamed skin is beta2-integrin/kindlin-3 dependent (Figure 2).

Proinflammatory, Th1, Th2, and Th17 cytokines are increased in the inflamed skin in KI mice

Beta2-integrins have previously been shown to have a role in restricting inflammation in various settings. However, the role

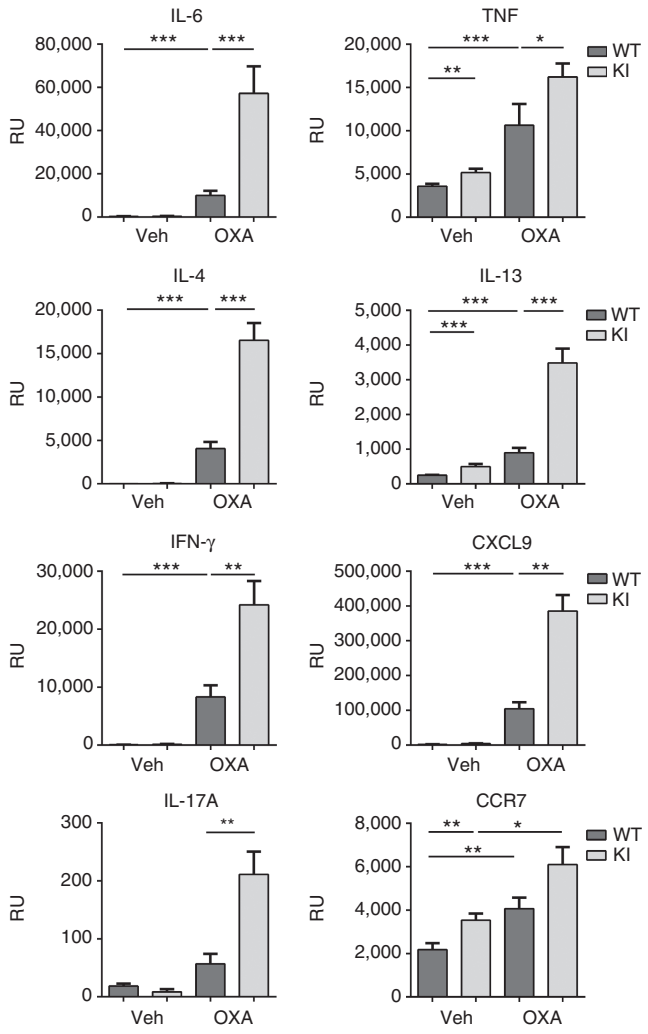

Figure 3. Increased inflammatory, Th1, Th2, and Th17 cytokines in inflamed skin in TTT/AAA-beta2-integrin knock-in (KI) mice. mRNA expression levels of proinflammatory (IL-6 and tumor necrosis factor), Th2 (IL-4 and IL-13), Th1 (IFN-γ and CXCL9), Th17 (IL-17) cytokines and of the chemokine receptor CCR7 were measured with quantitative real-time PCR after the CHS model in the ear tissue, $n = 8$. Mean ± SEM is shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. OXA, oxazolone; RU, relative unit; Veh, vehicle.

of functional integrins in regulating skin inflammation is unknown. We therefore investigated cytokine production during skin inflammation in the TTT/AAA-beta2-integrin KI mice. OXA induces both Th1 and Th2 cytokine production in the skin (Lehtimaki *et al.*, 2010). Although T-cell infiltration into the inflamed skin was reduced in KI mice (Figure 2), mRNA levels of proinflammatory cytokines (IL-6 and tumor necrosis factor), as well as Th1 (IFN-γ and CXCL9) and Th2 cytokines (IL-4 and IL-13) were upregulated in the OXA-challenged skin of KI mice as compared with WT mice (Figure 3). In addition, mRNA levels of IL-17 were increased in KI mice as compared with WT (Figure 3). The expression of chemokine receptor CCR7, which is known to be important in the migration of cutaneous DCs to skin-draining lymph nodes, was also increased in KI mice as compared with WT (Figure 3). These results show that functional integrins restrict the production of proinflammatory, Th1, Th2, and Th17 cytokines in the inflamed skin.

Cross-linking of IgE induces increased IL-4 expression in KI basophils/mast cells

As mast cell numbers were increased in uninflamed skin in KI mice (Figure 1b) and are therefore a putative source for increased Th2 cytokines in the inflamed skin of KI mice, we went on to investigate bone-marrow-derived cultured basophils/mast cells in KI and WT mice (Supplementary Figure S2 online). The cell culture included both basophils (FcepsilonRI+DX5+) and mast cells (FcepsilonRI+c-Kit+), and we found that KI basophils/mast cells produced more IL-4 mRNA in comparison to WT cells after cross-linking of DNP-specific IgE with specific antigen (Figure 4a). However, IL-6 mRNA expression levels were not statistically significantly increased in KI basophils/mast cells (Figure 4a). Cells were also stained with May-Grünwald-Giemsa to visualize mast cells and basophils (Figure 4b). IL-3–cultured KI mast cells and basophils were morphologically different from WT cells, looking more activated, which may partly explain their increased cytokine production.

The integrin/kindlin interaction restricts Th1 cell activation *in vivo*

We have previously shown that the integrin/kindlin interaction does not significantly affect T-cell activation in the spleen by WT, peptide-loaded DCs (Morrison *et al.*, 2013), and kindlin-3 is not required for T-cell trafficking into inflamed lymph nodes (Cohen *et al.*, 2013). This is in line with our results, which show similar $CD4^+$ and $CD8^+$ T–cell percentages in WT and KI inflamed skin-draining lymph nodes (LNs) (Figure 5a). Next, we investigated T-cell activation in inflamed lymph nodes. Interestingly, IFN-γ, CXCL9, and CXCL-10 levels were increased in skin-draining LNs of OXA-challenged mice, whereas IL-4 was not, indicating Th1 polarization of the immune response. Although IL-13 expression was increased in KI inflamed LNs, the expression levels were relatively low (Figure 5b). There was no statistically significant difference in the levels of the transcription factor for Th1 cells, T-bet, or the transcription factor for Th2 cells, GATA-3, in LN of KI mice in the CHS model (Figure 5b). Together, these results show that the beta2-integrin/kindlin interaction restricts Th1 cytokine production in inflamed lymph nodes.

One possible explanation for increased Th1 cytokine production in the draining lymph nodes of OXA-challenged TTT/AAA-beta2-integrin KI mice is through effects on antigen-presenting DCs. Migratory $CD11b^+$ DCs have previously been shown to be essential for IFN-γ production by $CD8^+$ and $CD4^+$ T cells in lymph nodes during skin inflammation (Tamoutounour *et al.*, 2013). In addition, we have shown that beta2-integrins restrict steady-state trafficking of DCs from the skin to the lymph nodes and Th1 polarization *in vivo* (Morrison *et al.*, 2014). However, the role of beta2-integrins in DC trafficking and activation under inflammatory conditions is poorly understood. We therefore investigated the phenotype and activation status of DCs in inflamed skin and

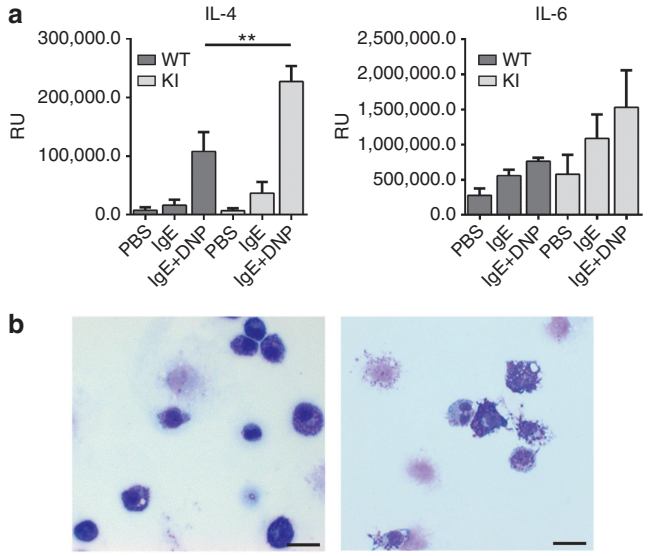

Figure 4. Increased IL-4 mRNA expression in TTT/AAA-beta2-integrin knock-in (KI) mast cells/basophils. (**a**) Mast cells/basophils were stimulated with anti-DNP IgE for 1 hour on ice and with DNP-human serum albumin for 6 hours. mRNA levels of IL-4 and IL-6 were measured with quantitative real-time PCR. $n = 3$. Mean ± SEM is shown. (**b**) Bone marrow cells were cultured for 13 days with IL-3 and stained with May-Grünwald-Giemsa to visualize mast cells and basophils. Bar = 20 μm. $^{**}P < 0.01$. KI, knock in; PBS, phosphate-buffered saline; RU, relative unit; WT, wild type.

draining lymph nodes. There were more DCs in both the inflamed skin and skin-draining lymph nodes of KI OXA-challenged mice (Figures 6a and b), whereas the number of macrophages was similar (Figure 6a). Further analysis showed that inflamed lymph node DCs in KI mice had a migratory phenotype (Miller *et al.*, 2012; Figure 6b) and that the activation marker CD80 was significantly elevated, whereas CD86 was expressed at equivalent levels in KI and WT mice (Figure 6c). These data therefore demonstrate that the integrin/kindlin-3 connection regulates Th1 cytokine production in inflamed lymph nodes, possibly through effects on DC survival, activation, and/or trafficking.

DISCUSSION

Leukocyte trafficking is known to be regulated by beta2-integrins. However, there is increasing evidence that these molecules also have essential roles in restricting inflammation in various settings. We have previously described that TTT/AAA-beta2-integrin KI mice, where the kindlin-3 binding site in the beta2-integrin cytoplasmic tail is disrupted, has dysfunctional beta2-integrins and a significant adhesion defect in leukocytes such as T cells and DCs. In addition, the TTT/AAA mutation affects beta2-integrin surface expression levels in leukocytes (Morrison *et al.*, 2013, 2014). Here, we used contact hypersensitivity as a model of skin inflammation to investigate the role of functional beta2-integrins in both leukocyte trafficking and inflammatory responses. Surprisingly, our results show that the kindlin-3/beta2-integrin interaction was not completely essential for neutrophil trafficking into the inflamed skin *in vivo*. However, T cells and eosinophils were dependent on this interaction for trafficking into the inflamed skin. We also show that functional integrins are essential for restricting inflammatory responses in the inflamed skin and lymph nodes.

Our results show that neutrophil trafficking into the inflamed skin *in vivo* is not completely dependent on beta2-integrin/kindlin-3. Although kindlin-3 KO neutrophils have been reported to have a severe adhesion defect (Moser *et al.*, 2009), we show here that kindlin-3 binding to the beta2-integrin tail is not strictly needed for neutrophil trafficking into the inflamed skin. However, as neutrophil numbers in the blood are significantly increased in the KI mice (Morrison *et al.*, 2013, 2014), there is a relative reduction in neutrophil accumulation in tissue. Therefore, we went on to study neutrophil adhesion under shear flow, a process that has previously been reported to be completely dependent on kindlin-3 (Lefort *et al.*, 2012). Surprisingly, neutrophil adhesion under shear flow conditions to ICAM-1/E-selectin/CXCL1 was similar in KI and WT neutrophils, whereas phorbol ester–induced KI neutrophil adhesion to ICAM-1 was severely compromised. In contrast, kindlin-3 has been previously reported to be essential for the transition of LFA-1 from the intermediate to the high-affinity state and for neutrophil arrest on ICAM-1/E-selectin/CXCL1 under similar flow conditions as those used here (Lefort *et al.*, 2012). Together, these results indicate that neutrophil adhesion under shear flow conditions and trafficking into sites of inflammation are dependent on kindlin-3 but are surprisingly not completely dependent on the binding of kindlin-3 to the beta2-integrin. Therefore, it appears that kindlin-3 may be able to regulate neutrophil adhesion through other mechanisms unrelated to its capability to bind to beta-integrin tails.

In contrast, T-cell trafficking into the OXA-challenged skin was reduced in TTT/AAA-beta2-integrin KI mice. This was not surprising as beta2-integrin KO mice have reduced $CD4^+$ and $CD8^+$ T–cell infiltration into OXA-challenged skin (Grabbe *et al.*, 2002), and kindlin-3 KO effector T cells cannot migrate into CFA-challenged skin (Cohen *et al.*, 2013). Our results confirm that T-cell trafficking into the inflamed skin is dependent on the beta2-integrin/kindlin-3 interaction. We speculate that the difference in the requirement for the kindlin/integrin interaction for neutrophils and T-cells trafficking into sites of inflammation might be due to additional E-selectin-mediated signals in neutrophils.

OXA induces Th1 cytokine production but also Th2 cytokine production in the inflamed skin (Lehtimaki *et al.*, 2010). We have shown here that $CD4^+$ T–cell trafficking into the inflamed skin is reduced in TTT/AAA-beta2-integrin KI mice, although Th1, Th2, and Th17 cytokines are upregulated, suggesting that neutrophils and mast cells (or other cell types) may be the source of increased cytokine levels in the inflamed skin. An important role for mast cells in the CHS responses has been shown by conditional depletion of mast cells, which resulted in a reduced CHS response and impaired skin DC migration/maturation and T-cell priming (Dudeck *et al.*, 2011). We show here that TTT/AAA-beta2-integrin KI mice have more mast cells in the uninflamed skin and increased DCs in the inflamed skin, and we also found increased IL-4 expression in cultured KI basophils/mast cells

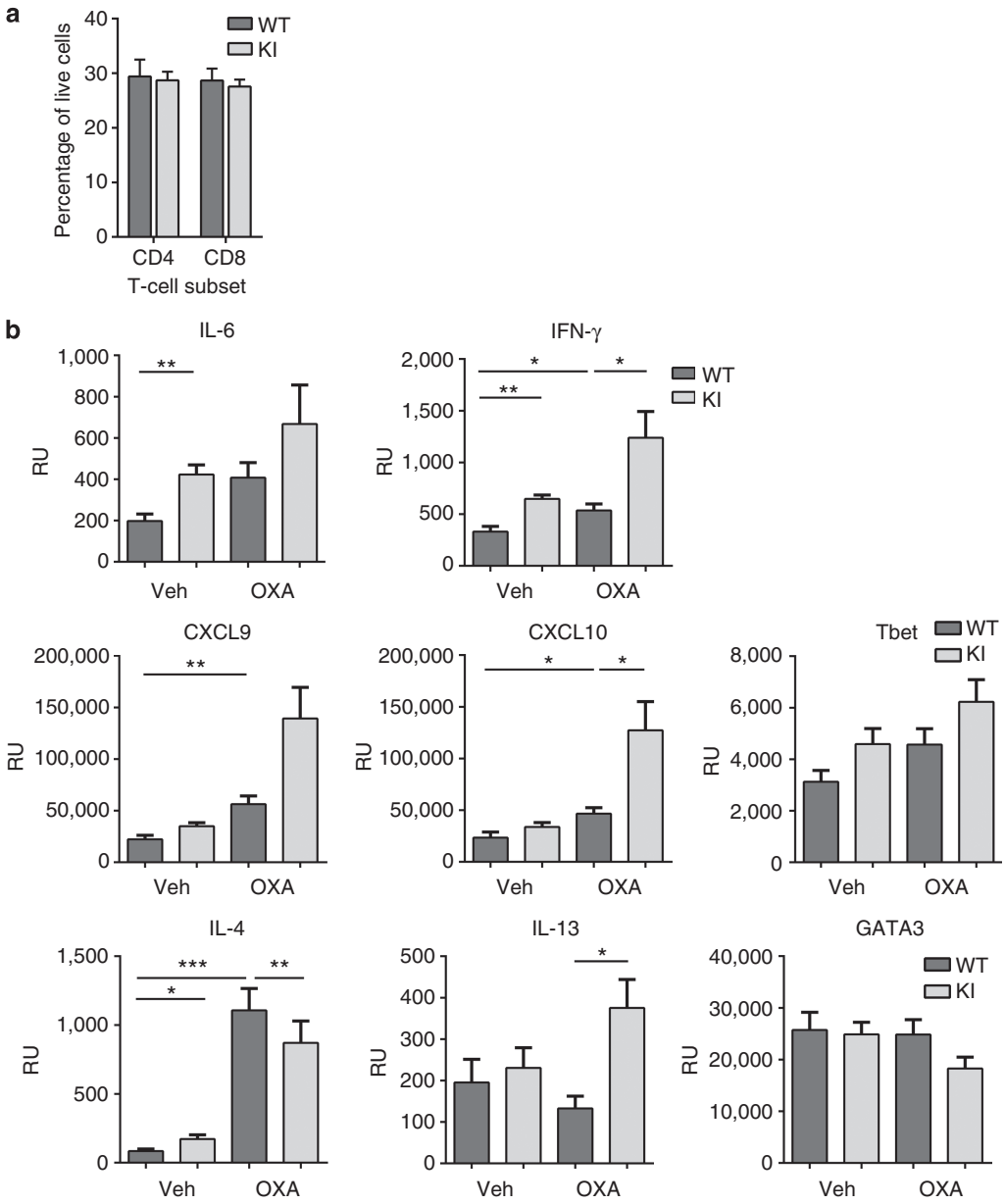

Figure 5. Normal T-cell subsets but increased T-cell cytokine responses in inflamed lymph nodes in TTT/AAA-beta2-integrin knock-in (KI) mice. (**a**) $CD4^+$ and $CD8^+$ T cells were quantified in lymph nodes by flow cytometry in wild-type (WT) and knock-in (KI) mice after the CHS model. $n = 5$. (**b**) mRNA expression of proinflammatory, Th1 cytokine, IFN-γ inducible chemokines, Th1 transcription factor T-bet, Th2 cytokines, and Th2 transcription factor GATA-3 in ear-draining lymph nodes after the CHS model was investigated with quantitative real-time PCR. $n = 8$. Mean ± SEM is shown. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. KI, knock in; OXA, oxazolone; Veh, vehicle; WT, wild type.

following IgE cross-linking. The beta2-integrin/kindlin-3 interaction may therefore limit mast cell numbers and function, and mast cells may be one source of the increased Th2 cytokine production in the skin of OXA-challenged KI mice. We have previously shown that beta2-integrin/kindlin-3/actin links restrict GM-CSF/IL-3 receptor signaling in myeloid cells (Morrison *et al.*, 2014). As mast cells/basophils are grown in the presence of IL-3, we suggest that beta2-integrin/kindlin-3 restricts signaling in DCs and mast cells through a similar mechanism. It is also possible that reduced surface expression of beta2-integrins in the TTT/AAA-beta2-integrin KI mice contributes to some of the inflammatory phenotypes shown here, although we have shown previously that rescuing surface expression of beta2-integrins by bafilomycin treatment in DCs does not rescue either their adhesion defect of DCs or their increased activation status (Morrison *et al.*, 2014).

We show here that there were increased IL-6 and IL-17 levels in the inflamed skin in mice with dysfunctional integrins. A similar phenotype of increased IL-17 levels has recently been reported in periodontal tissue in LAD-I patients and LFA-1 KO mice (Moutsopoulos *et al.*, 2014), where this

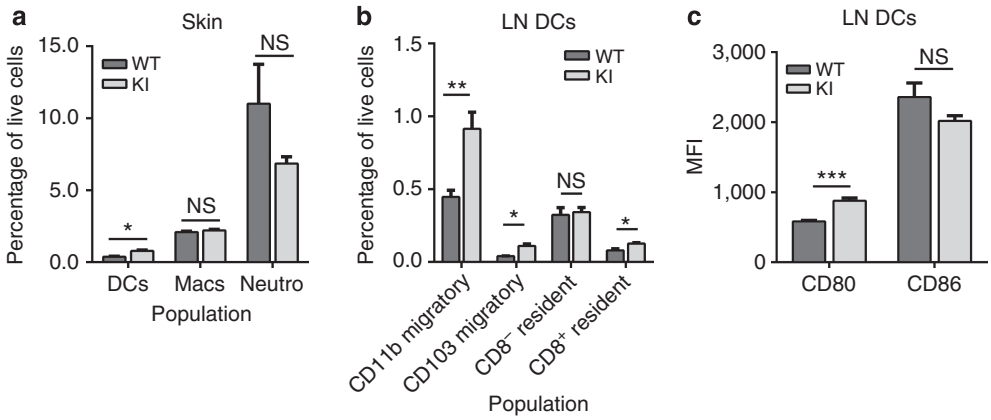

Figure 6. Increased dendritic cell (DC) activation and migratory phenotype in TTT/AAA-beta2-integrin knock in (KI) mice. The skin and ear-draining lymph node (LN) cells were dissociated after the CHS model and stained for flow cytometry. (**a**) Numbers of $CD11c^+$ major histocompatibility complex (MHC) class II^+ DCs, $F4/80^+$ macrophages, and $Gr\text{-}1^+$ neutrophils were quantified in oxazolone (OXA)-challenged skin of wild-type (WT) and KI mice. (**b**) Resident and migratory and DCs were quantified in WT and KI ear-draining lymph nodes after the CHS model. Resident ($CD8^+$ or $CD8^-$) DCs were determined as $CD3^-$ $CD19^-$ $B220^-$ $CD11c^+$ MHC class II^+. Migratory DCs ($CD103^+$ $CD11b^-$ or $CD103^-$ $CD11b^+$) were identified as $CD19^-$ $CD3^-$ $CD4^-$ $CD11c^+$ MHC class II^+. (**c**) Expression of CD80 and CD86 on total LN DCs was examined after the CHS model in WT and KI mice. $n = 5$. Mean ± SEM is shown. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. NS, non significant.

phenotype is thought to contribute to bacterial dysbiosis and inflammatory bone loss. Our findings therefore expand on the current knowledge of the role of beta2-integrins in regulating IL-17 responses *in vivo*.

Kindlin-3 has been previously reported to be dispensable for T-cell trafficking into inflamed lymph nodes (Cohen *et al.*, 2013), and here we did not detect any differences in $CD4^+$ or $CD8^+$ trafficking into inflamed lymph nodes in WT and KI mice. However, in the absence of the beta2-integrin/kindlin-3 interaction, IFN-γ and related cytokines were upregulated in both inflamed skin and inflamed lymph nodes in KI mice, whereas the levels of IL-4 were similar in inflamed lymph nodes, indicating Th1 polarization of the immune response. Interestingly, we found increased numbers of DCs in draining LNs after OXA challenge of KI mice, and these DCs displayed an increased activation state and a migratory phenotype, which may explain the increased T-cell activation and IFN-γ production in lymph nodes. In conjunction with our previous studies on restriction of DC migration by beta2-integrins under steady-state conditions (Morrison *et al.*, 2014), these findings show that beta2-integrins may also restrict DC migration from tissues to lymph nodes under inflammatory conditions.

In conclusion, we have here shown that, although the integrin/kindlin interaction is required for trafficking of certain leukocyte subtypes (T cells, eosinophils) into the inflamed skin, other leukocytes (surprisingly, neutrophils) are not dependent on the beta2-integrin/kindlin-3 interaction for trafficking into this inflamed site. In addition, we show that functional integrins have a major role in restricting inflammation in the skin, mast cell number and function, DC programming, and Th1 cytokine production in lymph nodes. These results significantly increase the current understanding of the role of beta2-integrins in inflammation and allergic skin diseases.

MATERIALS AND METHODS

Mice

Mice were maintained in the University of Helsinki, in compliance with Social and Health Services of the State Provincial Office of Southern Finland. The constitutive Itgb2 TTT/AAA KI mice have been previously described (Morrison *et al.*, 2013). C57/Bl6 mice were obtained from Charles River.

Contact hypersensitivity model

Mice were sensitized on the shaved backs with 1% OXA on day 0, and challenged with 0.3% OXA on both ears on day 5, followed by sample collection and analysis 24 hours later on day 6. Ear thickness was measured with a micrometer (Mitutoyo, Kanagawa, Japan) at 24 hours after challenge, after which ear dLNs and ears lobes were collected for RNA isolation, histological analysis, and flow cytometry.

Basophil/mast cell culture

Bone-marrow cells were isolated and cultured in RPMI for 13 days in the presence of IL-3 ($5\,ng\,ml^{-1}$) and 10% fetal calf serum as described earlier (Hida *et al.*, 2009). A total of 2×10^6 cells of WT and KI cells were stimulated with 10 μg of anti-DNP IgE (Sigma-Aldrich, St. Louis, MO) in 2 ml of culture medium for 1 h on ice. Cells were collected and resuspended in 2 ml of fresh medium with 200 ng of DNP-human serum albumin (Sigma-Aldrich) for 6 hours. Cells were lysed with lysis buffer included in the NucleoSpin RNA II Total RNA isolation kit (Macherey-Nagel, Germany), collected to flow cytometry or cytospin.

Histology

Paraffin-embedded skin sections of 2.5 μm were cut and stained with hematoxylin and eosin to detect neutrophils and eosinophils. Additional stainings were used to confirm the results obtained from hematoxylin and eosin staining. Anti-mouse Ly-6G (clone 1A8, Biolegend, San Diego, CA) was used to stain neutrophils, and Dominici staining was used to confirm eosinophil counts. Skin

sections were stained with o-toluidine blue for mast cell counts. For immunohistochemistry, ear specimens were embedded in Tissue-Tek oxacalcitriol compound (Sakura Finetek, Zoeterwoude, The Netherlands) and quick frozen on dry ice. Three-micrometer–thick frozen skin sections were fixed in acetone for 5 minutes and stained with monoclonal anti-CD3, anti-CD4, and anti-CD8 (BD Biosciences, San Jose, CA). Biotin-conjugated secondary antibodies (anti-rat IgG (H+L)) were from Vector Laboratories (Burlingame, CA). Cultured basophils/mast cells were collected for cytospin and slides stained with May-Grünwald-Giemsa.

RNA isolation and real-time PCR

Skin samples of WT and KI mice were homogenized with Ultra-Turrax T8 (IKA Labortechnik, Germany) in Trisure (Bioline, London, UK), and RNA was extracted according to the manufacturer's instructions and used as a template for cDNA synthesis. LN samples were homogenized in Lysing Matrix D tubes with Fast Prep, and the NucleoSpin RNA II Total RNA isolation kit (Macherey-Nagel) was used to isolate RNA from LN and cell samples. cDNA was synthesized from 0.5 μg of total RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Life Technologies, Carlsbad, CA). Taqman gene expression assay was performed with Taqman Fast Advanced Master Mix and quantitative real-time PCR with StepOnePlus System (Applied Biosystems) and StepOnePlus Software v2.3 (Applied Biosystems). Gene expression was normalized with 18 S rRNA, and the target gene expression was calculated by the comparative CT method (Applied Biosystems).

Flow cytometry

Single-cell suspensions of blood, bone marrow, and spleen from naive mice were stained with the following conjugated antibodies: Siglec F (E50-2440, BD Bioscience), Gr-1 (RB6-8C5, eBioscience, San Diego, CA), CD11c (HL3, eBioscience), CD11b (M1/70, eBioscience), and F4/80 (BM8, eBioscience). LNs and ear tissue were collected after the CHS model. LN samples were cut into small pieces and treated with 1 mg ml^{-1} collagenase (Life Technologies) and 2 mM EDTA at RT for 20 minutes. Ear tissue was collected on ice and treated with the MACS Miltenyi Whole skin dissociation kit (MACS Miltenyi Biotec, Germany). Skin specimens were incubated at +37 °C for 30 minutes in the presence of enzyme P, enzyme D, and enzyme A included in the Whole skin dissociation kit (MACS Miltenyi Biotec). Single-cell suspensions were stained with conjugated antibodies against CD103 (2E7), CD4 (RM4-5), CD11c (HL3), CD11b (M1/70), MHCII (M5/114.15.2), CD8a (53-6.7), CD19 (1D3), CD3 (145-2C11), Gr-1 (RB6-8C5), B220 (RA3-6B2), F4/80 (BM8), CD80 (16-10A1), and CD86 (GL1; all from eBioscience). Basophils/mast cells were stained with the following conjugated antibodies: FcεR1α (MAR-1, eBioscience), CD49b (DX5, eBioscience), and c-Kit (2B8, eBioscience). Fc block (clone 2.4G2, BD Biosciences) was included in all stains. Data were processed with the FlowJo Software (Tree Star, Ashland, OR).

Neutrophil adhesion under shear flow

Neutrophils were isolated from bone marrow by negative selection using a neutrophil isolation kit (MACS Miltenyi Biotec). Shear flow adhesion assay was performed as in Lek *et al.* (2013). Ibidi VI0.4 μ-slides (Germany) were coated with 6 μg ml^{-1} ICAM-1, 5 μg ml^{-1} CXCL1, and 30 μg ml^{-1} E-selectin (R&D Systems, Minneapolis, MN) or coated only with ICAM-1 overnight at 4 °C. Cells flowed over ICAM-1/CXCL1/E-selectin-coated VI 0.4 Ibidi μ-slides or ICAM-1-coated slides at a high (5 dynes cm^{-2}) continuous shear flow rate over a 5-min period. Alternatively, ICAM–1–coated slides were used together with 200 nM PdBu (Sigma-Aldrich), and the flow assay with neutrophils was performed 5 minutes after PdBu stimulation at a low shear flow rate of 0.3 dynes cm^{-2}. Cells were monitored by microscopy, and the number of adhered cells in the field of view was determined by manual counting.

Statistics

Student's two-tailed *t*-test, two-way analysis of the variance test, or the Mann–Whitney *U*-test (Graphpad Prism, La Jolla, CA) was used to calculate statistical significance.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Bai Y, Qian C, Qian L *et al.* (2012) Integrin CD11b negatively regulates TLR9-triggered dendritic cell cross-priming by upregulating microRNA-146a. *J Immunol* 188:5293–302

Balkow S, Heinz S, Schmidbauer P *et al.* (2010) LFA-1 activity state on dendritic cells regulates contact duration with T cells and promotes T-cell priming. *Blood* 116:1885–94

Cohen SJ, Gurevich I, Feigelson SW *et al.* (2013) The integrin co-activator Kindlin-3 is not required for lymphocyte diapedesis. *Blood* 122:2609–17

Dudeck A, Dudeck J, Scholten J *et al.* (2011) Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity* 34: 973–84

Etzioni A (2009) Genetic etiologies of leukocyte adhesion defects. *Curr Opin Immunol* 21:481–6

Grabbe S, Varga G, Beissert S *et al.* (2002) Beta2 integrins are required for skin homing of primed T cells but not for priming naive T cells. *J Clin Invest* 109:183–92

Hida S, Yamasaki S, Sakamoto Y *et al.* (2009) Fc receptor gamma-chain, a constitutive component of the IL-3 receptor, is required for IL-3-induced IL-4 production in basophils. *Nat Immunol* 10:214–22

Lefort CT, Rossaint J, Moser M *et al.* (2012) Distinct roles for talin-1 and kindlin-3 in LFA-1 extension and affinity regulation. *Blood* 119:4275–82

Lehtimaki S, Tillander S, Puustinen A *et al.* (2010) Absence of CCR4 exacerbates skin inflammation in an oxazolone-induced contact hypersensitivity model. *J Investig Dermatol Symp Proc* 130:2743–51

Lek HS, Morrison VL, Conneely M *et al.* (2013) The spontaneously adhesive leukocyte function-associated antigen-1 (LFA-1) integrin in effector T cells mediates rapid actin- and calmodulin-dependent adhesion strengthening to ligand under shear flow. *J Biol Chem* 288:14698–708

Malinin NL, Zhang L, Choi J *et al.* (2009) A point mutation in KINDLIN3 ablates activation of three integrin subfamilies in humans. *Nat Med* 15:313–8

Miller JC, Brown BD, Shay T *et al.* (2012) Deciphering the transcriptional network of the dendritic cell lineage. *Nat Immunol* 13:888–99

Morrison VL, James MJ, Grzes K *et al.* (2014) Loss of beta2-integrin-mediated cytoskeletal linkage reprogrammes dendritic cells to a mature migratory phenotype. *Nat Commun* 5:5359

Morrison VL, Macpherson M, Savinko T *et al.* (2013) The beta2 integrin-kindlin-3 interaction is essential for T-cell homing but dispensable for T-cell activation in vivo. *Blood* 122:1428–36

Moser M, Bauer M, Schmid S *et al.* (2009) Kindlin-3 is required for beta2 integrin-mediated leukocyte adhesion to endothelial cells. *Nat Med* 15:300–5

Moser M, Nieswandt B, Ussar S *et al.* (2008) Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat Med* 14:325–30

Moutsopoulos NM, Konkel J, Sarmadi M *et al.* (2014) Defective neutrophil recruitment in leukocyte adhesion deficiency type I disease causes local IL-17-driven inflammatory bone loss. *Sci Transl Med* 6:229ra40

Schmidt S, Moser M, Sperandio M (2013) The molecular basis of leukocyte recruitment and its deficiencies. *Mol Immunol* 55:49–58

Svensson L, Howarth K, McDowall A *et al.* (2009) Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. *Nat Med* 15:306–12

Tamoutounour S, Guilliams M, Montanana Sanchis F *et al.* (2013) Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity* 39: 925–38

Uzel G, Tng E, Rosenzweig SD *et al.* (2008) Reversion mutations in patients with leukocyte adhesion deficiency type-1 (LAD-1). *Blood* 111:209–18

Varga G, Balkow S, Wild MK *et al.* (2007) Active MAC-1 (CD11b/CD18) on DCs inhibits full T-cell activation. *Blood* 109:661–9