Loss of beta2-integrin-mediated cytoskeletal linkage reprograms dendritic cells to a mature migratory phenotype

Vicky Louise Morrison[1,2], Martyn John James[2], Katarzyna Grzes[1], Peter Cook[3], David Gavin Glass[4, 5], Terhi Savinko[2], Hwee San Lek[1], Christian Gawden-Bone[6], Colin Watts[6], Owain Richard Millington[4], Andrew Scott MacDonald[3], Susanna Carola Fagerholm[1,2]

[1]Medical Research Institute, Ninewells Hospital and Medical School, University of Dundee, Dundee, DD1 9SY, U.K.; [2]Institute of Biotechnology, University of Helsinki, Helsinki, Finland; [3]Manchester Collaborative Centre for Inflammation Research, University of Manchester, Manchester, M13 9NT, U.K.; [4]Strathclyde Institute for Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, G4 0RE, U.K.; [5]Institute of Photonics, SUPA, University of Strathclyde, Glasgow, G4 0NW, U.K.; [6]College of Life Sciences, University of Dundee, Dundee, DD1 5EH, U.K.

Address for correspondence: Susanna C Fagerholm (s.c.fagerholm@dundee.ac.uk; susanna.fagerholm@helsinki.fi). Institute of Biotechnology, P.O. Box 56, 00014 University of Helsinki

Running title: Integrins restrict migratory DC phenotype

Abstract

The actin cytoskeleton has been reported to restrict signaling in resting immune cells. Beta2-integrins, which mediate adhesion and cytoskeletal organization, are emerging as negative regulators of myeloid cell-mediated immune responses, but the molecular mechanisms involved are poorly understood. Here, we show that loss of the interaction between beta2-integrins and kindlin-3 abolishes the actin-linkage of integrins and the GM-CSF receptor in dendritic cells. This leads to increased GM-CSF receptor/Syk signaling, and to the induction of a transcriptional program characteristic of mature, migratory dendritic cells, accumulation of migratory dendritic cells in lymphoid organs, and increased Th1 immune responses *in vivo*. We observe increased GM-CSF responses and increased survival in neutrophils where the interaction between integrin and the cytoskeleton is disrupted. Thus, ligand-reinforced beta2-integrin tail interactions restrict cytokine receptor signaling, survival, maturation and migration in myeloid cells and thereby contribute to immune homeostasis *in vivo*.

Dendritic cells (DCs) function as the main antigen-presenting cells in the immune system. These cells are highly mobile and orchestrate immune responses by taking up antigen in the periphery before migrating to lymph nodes in both steady state and inflammatory conditions, where antigen is presented to T cells. The maturation or differentiation state of the DC determines the outcome of the immune response, and DCs control the initiation of helper, cytolytic and regulatory T cell responses *in vivo*.

Beta2-integrin adhesion receptors are expressed in both T cells and DCs where they play strikingly different roles in immune regulation. In T cells, beta2-integrins and their regulation by talin and kindlin-3 are required for firm adhesion under conditions of shear flow, and therefore for T cell trafficking into lymph nodes and inflammatory sites [1-4]. Talin (but not kindlin-3) is required for efficient T cell activation *in vitro* and *in vivo* [1,2,5]. In DCs, beta2-integrins play a very different role, as they are not required for DC migration to lymph nodes in steady state [6]. Instead, beta2-integrins have been described to restrict DC-mediated priming of T cells [7-9] and Th17 differentiation [10], although the molecular mechanisms involved have remained unclear. Beta2-integrins have been reported to play a regulatory role in macrophages by restricting Toll-like receptor (TLR) signaling through pathways dependent on Syk and IL-10 [11,12], but have also recently been reported to promote TLR4 signaling in endosomes in DCs and DC-mediated T cell activation [13]. Therefore, both the roles of beta2-integrins in DC signaling and the molecular mechanisms involved in regulation of DC-mediated immune responses by beta2-integrins remain unclear.

Integrins integrate the cell exterior to the actin cytoskeleton, and integrin activation and signaling is regulated by the binding of cytoplasmic proteins, such as talin and kindlin-3, to the beta2-integrin cytoplasmic domain [14-18]. Kindlin-3 is an essential integrin regulator which binds to the integrin beta-chain cytoplasmic tail through a membrane-distal NPXY domain [19] and a TTT-domain [1]. A deficiency in kindlin-3 causes Leukocyte Adhesion Deficiency type III (LAD-III), a disorder where beta2- and beta3-integrins cannot mediate leukocyte and platelet adhesion. LAD-III patients have a similar phenotype to patients with beta2-integrin subunit mutations (LAD-I), in that they suffer recurrent infections, with the addition of severe bleeding due to loss of platelet adhesion [16,20].

Recently, the actin cytoskeleton has been implicated in restriction of signaling in resting immune cells, by causing compartmentalization of the plasma membrane and thereby restricting mobility of cell surface receptors and associated molecules [21]. Whether integrins, the main

“integrators” of cell exterior and actin cytoskeleton in cells, contribute to this process has remained unclear.

Here, we show that in the absence of beta2-integrins or beta2-integrin-kindlin-3-mediated cytoskeletal organization, bone marrow-derived DCs (BMDCs) display loss of association of the GM-CSF receptor with the actin cytoskeleton, increased GM-CSF receptor signaling and a maturation phenotype characteristic of *in vivo* migratory DCs, which leads to increased Th1 priming. Our data reveal a novel function of ligand-reinforced beta2-integrin-tail interactions in the restriction of cytokine receptor signaling of myeloid cells, and of myeloid cell survival, maturation and migration *in vivo.*

Results

The integrin-kindlin link in DCs restricts T cell activation

To investigate beta2-integrin-mediated regulation of immune responses, we used a mouse model which has recently been described where the kindlin-3 binding site in the beta2-integrin cytoplasmic domain has been mutated (beta2$^{TTT/AAA}$ integrin knock-in mice) [1]. We have previously investigated the activation of T cells from beta2$^{TTT/AAA}$ integrin knock-in mice, using *in vitro* models and *in vivo* adoptive transfer systems with WT DCs to prime T cell responses, and found knock-in T cell activation to be normal[1]. To investigate the role of the integrin-kindlin-3 interaction in DC-mediated immune responses *in vivo*, beta2$^{TTT/AAA}$ integrin knock-in mice were immunized with peptide antigen in Complete Freund's Adjuvant (CFA) to induce T cell activation by beta2$^{TTT/AAA}$ integrin knock-in DCs. Strikingly, we found that the CD4 T cell response generated in both the spleen and the draining (inguinal) lymph nodes of beta2$^{TTT/AAA}$ integrin knock-in mice was elevated compared to WT mice (Figure 1A). This result led us to investigate the phenotype of beta2$^{TTT/AAA}$ integrin knock-in DCs.

We assessed the T cell priming ability of knock-in BMDCs in a co-culture system with TCR transgenic CD4 T cells specific for the ovalbumin (OVA) 323-339 peptide (OT-II T cells). Resting (non-TLR activated) peptide-loaded beta2$^{TTT/AAA}$-integrin knock-in DCs induced higher levels of OT-II T cell activation *in vitro*, as measured by T cell expression of the activation markers CD69, CD25, and CD44 (Figure 1B). Using optical trapping, we show that knock-in DCs formed weaker antigen-dependent contacts with OT-II T cells, at both the initial contact (2min) and at longer (24hr) contact times, compared to WT cells (Figure 1C). This reduced adhesion strength is likely due to impaired binding of the integrin ligand ICAM-1 on the T cell surface by DC beta2-integrins, as the kindlin-integrin interaction has been described to regulate integrin adhesion to ligands [1]. Indeed, knock-in DCs showed reduced binding to the beta2-integrin ligands ICAM-1 and iC3b (Figure 1D), as well as to culture plates (Figure 1E), demonstrating the general importance of beta2-integrin-kindlin-3 interactions in integrin-mediated cellular adhesion. As has been reported for other cell types carrying the TTT/AAA mutation [1], surface expression of beta2-integrins was lower in knock-in DCs (Supplementary Figure S1A), likely due to a deficiency in sorting nexin binding to the mutated integrin, leading to deficient integrin recycling from endosomes [22]. Indeed, treatment of cells with a lysosome inhibitor, bafilomycin, completely rescued the integrin surface expression defect in beta2$^{TTT/AAA}$ integrin-expressing cells (Supplementary Figure S1B). However, bafilomycin treatment did not rescue

cell adhesion to ICAM-1 (Supplementary Figure S1C), confirming that the defect in DC adhesion is due to impaired integrin function rather than a deficiency in integrin surface expression.

Next we investigated CD4 T cell activation *in vivo* using MHC class II tetramers following the adoptive transfer of either WT or knock-in LPS-activated peptide-loaded DCs into WT recipient mice. In this system, only the DCs carry the TTT/AAA mutation whilst all other cell types are WT. In agreement with the *in vitro* data, beta2$^{TTT/AAA}$-integrin knock-in DCs triggered a larger T cell response *in vivo* in the spleen than WT DCs (Figure 1F), although as this experiment used small amounts of adoptively transferred DC, which have to survive and migrate in the WT hosts, the CD4 T cell response was not upregulated as much as in Figure 1A, as expected. Taken together, these results suggest that beta2$^{TTT/AAA}$-integrin knock-in DCs are pre-disposed to activate T cells without TLR stimulation, driving elevated T cell activation *in vitro* and *in vivo*, despite showing reduced adhesion to integrin ligands and forming weaker antigen-dependent contacts with T cells.

Beta2-integrin knock-in DCs display elevated maturation

Our results show that adhesion-deficient beta2$^{TTT/AAA}$-integrin knock-in DCs drive increased T cell immune responses *in vitro* and *in vivo*. To further investigate the phenotype of knock-in BMDCs, expression of surface markers was analyzed by flow cytometry. Surprisingly, expression of MHC class II and co-stimulatory molecules CD80, CD86 and CD40 was higher in GM-CSF-cultured, as well as LPS-activated, knock-in DCs compared to WT cells (Figure 2A-B). Interestingly, expression of these markers showed a dose-dependent increase in response to GM-CSF (Supplementary Figure S2A). Bafilomycin treatment of the knock-in DCs, which rescued cell surface expression of TTT/AAA integrins, did not inhibit these increased responses to GM-CSF (Supplementary Figure S1D), demonstrating that the decreased surface expression of integrins in knock-in DCs was not responsible for their active phenotype. In terms of cytokine production by knock-in DCs, IL-12p40 was significantly increased with GM-CSF stimulation, whilst IL-12p40, IL-12p70, IL-10, IL-23p19, TNF-alpha and nitric oxide were elevated following LPS stimulation (Figure 2C and Supplementary Figure S2B), indicating that GM-CSF and TLR responses were elevated in knock-in cells.

As cellular responses to GM-CSF were elevated in beta2$^{TTT/AAA}$ integrin knock-in DCs, we went on to investigate GM-CSF signaling in knock-in BMDCs. Surface expression of the GM-CSF receptor was slightly increased in knock-in DCs compared to WT cells (Figure 2D). The GM-CSF

receptor signals through the JAK/STAT pathway leading to activation of further downstream pathways such as MAPK and Akt. Although initial JAK2/STAT5 activation after GM-CSF stimulation was similar in WT and KI cells (Figure 2E), GM-CSF signaling was sustained in knock-in DCs, which displayed increased levels of p-MAPK, p-p38 and p-Akt downstream of JAK/STAT signaling at a 3h timepoint after GM-CSF addition (Figure 2F). Syk has been previously shown to be downstream of beta-common chain-containing cytokine receptor signaling [23], and we found here that Syk signaling was also up-regulated in beta2$^{TTT/AAA}$ integrin-expressing cells downstream of the GM-CSF receptor (Figure 2F). TLR (LPS) stimulation did not further increase Syk signaling in these cells, but LPS stimulation did result in up-regulation of NFκB and Akt signaling in knock-in DCs, whilst levels of p-p38 and pErk after LPS stimulation were similar in WT and knock-in cells (Figure 2G). These results demonstrate that GM-CSF (and TLR) signaling is up-regulated in DCs where the kindlin-3-integrin link has been disrupted.

Knock-in neutrophils show increased responsiveness to GM-CSF

To investigate whether increased GM-CSF signaling was a general feature in the beta2$^{TTT/AAA}$ integrin knock-in mice, we investigated neutrophil GM-CSF responses. Neutrophilia is a significant symptom in murine models with defects in adhesion molecules, including the recently developed beta2$^{TTT/AAA}$ integrin knock-in mouse [1,24-27]. In addition to the previously reported increase in neutrophil numbers in the bloodstream of these mice [1], we found neutrophil numbers in the bone marrow of beta2$^{TTT/AAA}$ integrin knock-in mice to be significantly elevated compared to WT controls (Supplementary Figure S3A). As GM-CSF is known to be vital for neutrophil survival [28], we tested the survival capacity of WT and knock-in neutrophils *in vitro*. The results show significantly increased *ex vivo* survival of knock-in neutrophils, both in the absence of GM-CSF, likely reflecting continuing cellular responses to endogenous signals from their environment within tissues of the mice, and in the presence of GM-CSF (Supplementary Figure S3B). Knock-in neutrophils also showed increased GM-CSF signaling (Supplementary Figure S3C). These data demonstrate a more general role for beta2-integrins in the regulation of GM-CSF signaling in leukocytes.

Knock-in bone marrow cells show elevated IL-3 response

Because the GM-CSF receptor shares the beta-common chain with the IL-3 and IL-5 cytokine receptors, we went on to investigate IL-3 responses in myeloid leukocytes. Myeloid leukocytes survived in culture without IL-3, but interestingly, beta2-integrin knock-in bone marrow myeloid cell proliferation in response to IL-3 was significantly increased compared to WT cells, with a greater effect seen at highest IL-3 concentrations (day 4 average MFIs for CFSE in KI cell cultures are 171 at 0.1ng/ml IL-3; 135 at 1ng/ml IL-3; 110 at 10ng/ml IL-3), although the effects on survival were not very substantial (Supplementary Figure S3D). These data indicate that beta2-integrins are especially important in the regulation of GM-CSF receptor (and TLR) signaling. In contrast, Flt3 ligand-cultivated DCs and M-CSF-cultured macrophages did not display increased activation responses (Supplementary Figure S4A-B). Collectively, these results suggest that beta2$^{TTT/AAA}$ integrin knock-in myeloid cells are more responsive to GM-CSF/IL-3 family of cytokines than WT cells.

Beta2-integrin knock-in DCs have a mature migratory phenotype

Our data imply that maturation status is affected in adhesion-deficient beta2$^{TTT/AAA}$-integrin knock-in DCs. To investigate the global effects on gene transcription in these cells, we performed next generation sequencing (RNAseq) from WT and beta2$^{TTT/AAA}$ integrin knock-in BMDCs to map the transcriptomes. The data reveal that there were significant differences in gene expression between WT and knock-in DCs (Figure 3A-B and Supplementary Datasets S1-2). Co-stimulatory molecules CD86 and CD40 and cytokines such as IL-12 were confirmed to be upregulated on the transcriptional level in knock-in cells (Figure 3B). Also, molecules involved in antigen presentation (MHC class II and CIITA), and in the JAK/STAT signaling (JAK2, STAT5, SOCS2) pathway were upregulated, whilst components involved in antigen uptake (Sort1, CD68, Stab1) were downregulated in knock-in cells (Figure 3B), confirming that the maturation status was indeed affected in knock-in DCs. However, antigen uptake was not reduced in knock-in cells (Figure 3C), indicating that the cells can still take up antigen although they are in a more mature state.

Crucially, CCR7, which is essential for DC migration to lymph nodes, was found to be significantly upregulated in knock-in DCs, as determined by next generation sequencing (Figure 3B) and flow cytometry (Figure 3D). By comparing gene expression profiles to previously described profiles of DC populations *in vivo* [29], it became apparent that there was a significant overlap of the transcriptomes of knock-in DC and *in vivo* migratory DC populations (non-lymphoid tissue cDCs that

have migrated to lymph nodes) (Figure 3E and Supplementary Dataset S3). In migratory DCs, beta2-integrins (*Itgb2* and *Itgax* genes) are downregulated, whilst CCR7 is upregulated; the same is true in knock-in DC (where beta2-integrins also display reduced adhesiveness due to the TTT/AAA mutation). Functional annotational clustering analysis[30] of genes which were upregulated in both migratory and knock-in DCs further (Supplementary Dataset S3) further revealed that genes encoding for proteins involved in small GTPase signal transduction (such as Arhgef40, Rabgap1l, Net1, Rap2b), zinc-finger containing proteins (Cbl-b, Trim24 etc) and certain cell adhesion proteins (VCAM-1) were common to both types of cells. In contrast, genes encoding for proteins of certain other cellular pathways, such as macromolecule catabolic processes (some ubiquitin ligases and caspases), were not upregulated in KI DCs but were upregulated in migratory cells. Of the genes that were upregulated in both migratory and KI DCs, small GTPases such as Rap2 and cell adhesion proteins such as VCAM-1 are well known to regulate cell migration, and Cbl-b has been previously described as a negative regulator of beta2-integrin-mediated adhesion [31]. Therefore, it appears that the non-adherent knock-in DCs had switched on a transcriptional program characteristic of DC populations which have matured and migrated to lymph nodes. Thus, the integrin-kindlin link, which is essential for DC adhesion to the environment, is crucial for restricting the DC maturation program.

To investigate whether switching on a transcriptional program characteristic of migratory DCs influenced knock-in DC behavior *in vivo*, we investigated numbers of DCs in lymphoid organs in beta2$^{TTT/AAA}$-integrin knock-in mice. We found that there were indeed significantly increased numbers of CD11c+ MHC class II+ DCs in the spleen and peripheral lymph nodes of knock-in animals compared to WT controls (Figure 4A). Specifically, numbers of migratory CD11b+ and CD103+ DCs were increased in knock-in lymphoid tissues, whereas numbers of CD8+ and CD8- resident DCs were unaffected by the TTT/AAA mutation (Figure 4B). T and B cell numbers in lymph nodes were normal in naïve knock-in mice[1] and in immunized mice (Figure 4C), but DC numbers in lymph nodes were increased in both resting and immunized knock-in mice (Figure 4D). In addition, knock-in splenic DCs from both resting and immunized mice displayed significantly increased expression of CD86 *in vivo*, and a trend towards elevated CD40 expression, although this did not reach statistical significance (Figure 4E). Importantly, following administration of fluorescent OVA antigen into the skin, we found increased numbers of OVA+ DCs in the draining lymph nodes 24hrs later (Figure 4F). Therefore, loss

of the beta2-integrin-kindlin-3 link in DCs induces a switch to a mature phenotype *in vitro* and *in vivo*, and induced DC migration to lymph nodes and/or increased survival of KI DCs.

Integrin-kindlin-actin links regulate DC maturation

Maturation of DCs is well known to be associated with reduced adhesion and changes in cell morphology[32], and we found that LPS maturation of DCs indeed significantly reduced adhesion to integrin ligands (Figure 5A). To investigate whether loss of functional integrins was responsible for induction of DC maturation in beta2$^{TTT/AAA}$ integrin knock-in cells, we analyzed signaling and maturation status in beta2-integrin null DCs. Similar to the beta2$^{TTT/AAA}$ integrin knock-in DCs, p-Syk and p-Akt levels were elevated in GM-CSF-stimulated beta2-integrin null DCs (Figure 5B). These cells also expressed more MHC class II, CD80, CD86 and CD40 (Figure 5C), and produced more IL-12p40 (in response to GM-CSF and LPS) and IL-10 (in response to LPS) than WT cells (Figure 5D). These data indicate that the elevated signaling in knock-in DCs is due to the absence of functional beta2-integrins.

We next directly assessed the role of integrin-ligand interactions in the regulation of signaling and maturation of DCs by comparing adhered WT DCs to WT DCs which were detached and placed in tubes. The results show that p-Syk and p-p38 levels were elevated in the detached cells compared to adhered cells (Figure 5E). The expression of the co-stimulatory markers CD80, CD86, CD40 and MHC class II by WT DCs was significantly increased in detached cells compared to adhered cells when stimulated with GM-CSF (Figure 5F). Additionally, production of IL-12p40 and IL-10 was increased in detached cells compared to adhered cells following GM-CSF and LPS stimulation, respectively (Figure 5G). Together, these results indicate that beta2-integrin-mediated ligand binding regulates signaling and maturation in myeloid DCs. To confirm this, we investigated IL-12 production (Figure 5H) and CD86 expression (Figure 5I) by WT and knock-in DCs plated on different substrates, e.g. beta2-integrin substrates (petri dishes and ICAM-1), a beta1-integrin substrate (fibronectin) and tissue culture plates (unspecific substrate). As the knock-in mutation is in the beta2-integrin tail, knock-in DCs are not expected to display any abnormality when plated on a beta1-integrin substrate. Indeed, knock-in cells on beta2-integrin substrates displayed elevated maturation, whilst knock-in DCs on beta1-integrin substrates displayed similar activation to WT cells (Figure 5H-I). These results indicate that WT beta2-integrin-mediated ligand binding and/or ligand-reinforced integrin tail

interactions provides suppressory signals to the GM-CSF receptor complex, which are lost in KI DCs, and that ligand-induced beta1-integrin-mediated signals can compensate for the deficiency in function of beta2-integrins in KI cells.

To directly assess the role of adhesion (rather than integrin-kindlin-3 contacts) in regulation of DC signaling, we further investigated whether Mn^{2+}, a traditional extracellular activator of integrin-mediated adhesion, could rescue KI DC adhesion and at the same time suppress signaling. Although Mn^{2+} treatment of cells did not result in a formal rescue of adhesion, low amounts of Mn^{2+} did result in a slight increase in KI DC adhesion to ICAM-1 and iC3b (Supplementary Figure S5A), but IL-12 production and CD86 expression were increased rather than decreased after Mn^{2+} treatment (Supplementary Figure S5B). These results show that increased adhesion alone, without integrin/kindlin links, was not sufficient to reduce GM-CSF receptor signaling in cells; rather, the integrin/kindlin link is required for integrins to suppress GM-CSF receptor signaling in DCs.

The cortical actin cytoskeleton is known to restrict receptor movement and signaling in immune cells [21]. Integrin cytoplasmic domains are important in linking the plasma membrane to the actin cytoskeleton, and therefore play pivotal roles in actin organization. In addition, the integrin TTT/AAA mutation significantly perturbs integrin-mediated actin reorganization in cells [1,14]. Therefore, we investigated the subcellular localization of the beta2-integrins, kindlin-3 and the GM-CSF receptor in DCs. A significant amount of beta2-integrin, kindlin-3, talin and the GM-CSF receptor were localized to the cytoskeletal fraction of WT DCs, but the TTT/AAA-mutation of the integrin abolished these associations (Figure 6A), indicating that the integrin-kindlin link is essential for actin recruitment of beta2-integrins and cytokine receptors. Levels of another membrane receptor, CD9, (a tetraspanin which mediates MHC II lateral associations in DCs[33]) were equivalent in WT and knock-in cytoskeletal fractions (Figure 6A), indicating that not all cell surface receptors are regulated in this way. To test whether the loss of the integrin-actin link is responsible for the increased signaling in knock-in DCs, cells were treated with cytochalasin D to disrupt actin filaments. Cytochalasin D treatment of WT DCs resulted in up-regulation of p-Syk and p-MAPK (Figure 6B), as well as an increase in CD86 and CD40 expression (Figure 6C), and in IL-12p40 production (Figure 6D). Together, these data indicate that ligand-reinforced integrin-actin interactions, regulated through kindlin-3, are necessary for the regulation of GM-CSF receptor signaling in DCs, and for the regulation of downstream effects including DC maturation.

Syk is partly responsible for the mature DC phenotype

Levels of p-Syk were elevated in beta2$^{TTT/AAA}$-integrin knock-in DCs (Figure 2F). To investigate the role of Syk in the elevated DC activation status we made use of the selective Syk inhibitor II [34] and a p38 inhibitor (SB203580). Syk inhibition of WT DCs in suspension resulted in a reduction of both co-stimulatory marker expression (Figure 7A) and cytokine production (Figure 7B), confirming that these activation properties of DCs are controlled by GM-CSF-induced Syk activity. Meanwhile, p38 inhibition in WT DCs reduced CD80 expression levels (Figure 7A) and IL-10 production (Figure 7B), but not CD86, CD40, MHC class II or IL-12p40 (Figure 7A-B), suggesting that the p38 signaling pathway downstream of Syk regulates CD80 and IL-10 in GM-CSF-stimulated DCs. In addition, Syk inhibition in both beta2-integrin knock-in and null DCs resulted in a partial reduction of IL-12p40 and IL-10 production (Figure 7C), showing that the active phenotype of knock-in DCs is due, in part, to elevated Syk activity. Together, these results reveal that integrin/kindlin-mediated signals negatively regulates downstream GM-CSF receptor signaling in DCs, mediated through Syk, which in turn regulates the expression of co-stimulatory molecules and production of cytokines.

Beta2-integrin knock-in DCs induce elevated Th1 responses

"Muted" DC activation has been associated with Th2 responses, whilst conventionally matured (TLR-activated) DCs generally promote Th1/Th17 responses [35]. In addition, Syk signaling in DCs is essential for Th1/Th17 responses *in vivo* [36,37] To investigate whether beta2$^{TTT/AAA}$ integrin knock-in DCs specifically induce Th1/Th17 responses *in vivo*, WT or knock-in DCs were loaded with antigens that have previously been shown to promote differential T helper cell subset polarization following DC adoptive transfer [38]; the Th2 schistosome soluble egg antigen (SEA), and heat-killed *Propionibacterium acnes* which drives Th1/Th17 responses (Supplementary Figure S6A-B). Beta2$^{TTT/AAA}$ integrin knock-in DCs induced greater antigen-specific IFNγ production by T cells in both immunization groups (Figure 8A), indicative of a stronger Th1 response. Conversely, the Th2 response (identified by IL-4, IL-5, IL-13, and IL-10) was significantly lower in the knock-in DC immunization groups, especially for the SEA group, which promoted the largest Th2 response (Figure 8A). IL-17, indicative of a Th17 response, was only produced at marked levels in the *P. acnes* antigen group, where production was equivalent in the WT and knock-in DC immunization groups (Figure 8A).

Polyclonal stimulation confirmed the increased Th1 priming and impaired Th2 responses (Supplementary Figure S6C).

We found increased production of CXCL10, a chemokine that promotes Th1 cell differentiation [39], by knock-in DCs compared to WT DCs (Figure 8B), which, together with the increased IL-12 production by these cells, may account for the elevated priming of Th1 responses by knock-in DCs *in vivo*. These data therefore demonstrate that beta2$^{TTT/AAA}$ integrin knock-in DCs, which show increased signaling and an elevated activation state associated with migratory DCs, specifically promote elevated Th1 responses, and are strikingly impaired in their priming of Th2 responses, *in vivo.*

In conclusion, we show that specific ligand-reinforced integrin tail interactions and integrin-mediated cytoskeletal organization play an unexpected role in restricting myeloid cell signaling and activation, and the maturation program characteristic of migratory DCs, thereby regulating T cell immune responses *in vivo*.

Discussion

Beta2-integrins are emerging as important negative regulators of immune cell signaling, especially in myeloid cells, in stark contrast to the mainly positive role that integrins play in growth factor receptor signaling in other cell types. As such, beta2-integrins have been described to negatively regulate TLR and interferon signaling in macrophages [11,12]. In addition, active beta2-integrins in DCs and macrophages inhibit their priming of T cells [7]. Mechanisms by which beta2-integrins negatively regulate signaling are emerging. CD11b-induced Syk activation has been proposed as a mechanism to negatively regulate TLR signaling in macrophages [11], and indirect mechanisms involving the signaling inhibitor A20 and the anti-inflammatory cytokine IL-10 have also been described [12]. However, the CD11b-integrin has also recently been reported to instead *positively* regulate TLR4 signaling in endosomes in DCs (as opposed to macrophages), and to be required for TLR4-triggered responses *in vivo* [13]. In contrast, in this study, we describe that ligand-reinforced beta2-integrin tail interactions with kindlin-3 and cytoskeletal coupling negatively regulate DC maturation through restricting GM-CSF receptor signaling. Therefore, in DCs, ablating beta2-integrin-mediated cytoskeletal organization (through mutation of the kindlin-binding site in the integrin, or beta2-integrins themselves) results in a strikingly different phenotype than ablating the CD11b integrin subunit alone, likely reflecting redundancy between different beta2-integrins in DCs, as DCs express (at least) CD11a- and CD11c-chains in addition to CD11b.

Interestingly, we found that the integrin/kindlin connection also restricts GM-CSF receptor signaling in neutrophils. Neutrophilia is a significant symptom in murine models with defects in adhesion molecules [1,24-27]; our data suggests that increased GM-CSF receptor signaling in these cells may contribute to the significant neutrophilia found in these mouse models.

Intriguingly, ablating the beta2-integrin/kindlin-3 interaction - results in DC maturation to a migratory phenotype, increased DC trafficking to lymph nodes, and increased T cell priming *in vivo*. Reprogramming of DCs to a mature, migratory phenotype by loss of integrin/kindlin links implies that beta2-integrins contribute to immune homeostasis by restricting DC maturation in tissues until migration and maturation is required. Does loss of ligand-induced integrin signals contribute to DC maturation/migration also under physiological conditions? Interestingly, migratory DCs found in lymph nodes express less beta2-integrins than their tissue counterparts (Supplementary Dataset S3) [29], indicating that under steady state conditions, beta2-integrins are indeed down-regulated when cells

migrate to lymph nodes, possibly through a cell-intrinsic effect during the DC life cycle. We speculate that beta2-integrins, which are not required for DC migration to lymph nodes in steady state [6], instead mediate DC interactions with their extracellular environment in peripheral tissues, which *restricts* their migration to lymph nodes. Loss of ligand-binding due to integrin down-regulation reprograms DCs and contributes to migration to lymph nodes under steady-state conditions. In addition, it is well described that during DC maturation due to external stimuli, such as TLR stimulation, adhesion is downregulated and the cell morphology and actin cytoskeleton is fundamentally altered [32,40], and the cells become more migratory[40], and we show here that adhesion to integrin-ligands is indeed reduced in LPS-matured DCs. Therefore, maturation-induced loss of ligand-binding and/or cytoskeletal coupling may promote DCs to switch on the transcriptional program that allows for their migration to lymph nodes and for the subsequent activation of T cells.

The elevated maturation status in DCs lacking beta2-integrin-mediated cytoskeletal organization was mediated by increased GM-CSF receptor signaling. The membrane skeleton is essential for controlling signaling in resting immune cells [21,41] and integrins act as links or integrators between the cell exterior and the cell cytoskeleton in cells [42]. They would therefore be well positioned for forming “pickets” in the “picket-fence” model of the plasma membrane, and for restricting movement of receptors in the plasma membrane [43]. We propose that ligand-reinforced integrin-kindlin- linkages fundamentally restrict the movement of the actin-linked GM-CSF receptor (and possibly other receptors or adapter proteins) at the DC plasma membrane, thereby limiting downstream signaling, a function of integrins in immune cells that has not previously been appreciated (Supplementary Figure S8). We further show that increased GM-CSF receptor-induced Syk signaling is, in part, responsible for the elevated activation phenotype in these knock-in DCs. TLR responses were also elevated in knock-in DCs, although TLR signaling did not further activate Syk in these cells. Interestingly, a complex between the beta-common chain of IL-3/IL-5/GM-CSF receptors and the ITAM-containing Fc receptor γ-chain has been previously described, leading to recruitment of Syk to the IL-3 receptor in basophils [23]; a similar mechanism may be at play in DCs and neutrophils. The exact mechanism(s) of integrin-mediated regulation of signaling receptors in DCs need to be established in future studies.

A striking result from this work was that beta2$^{TTT/AAA}$-integrin knock-in DCs, which displayed an elevated activation state, induced elevated Th1 responses *in vivo*, whilst Th2 priming was

impaired. Indeed, highly activated DCs have previously been described to preferentially promote a Th1 response[44], whilst Th2 responses are generally associated with a "muted" DC activation state [35]. In addition, Syk signaling in DCs has been previously reported to be essential for Th1 responses *in vivo* [36,37]. We show that increased Syk signaling resulted in elevated DC activation and increased IL-12 production by knock-in DCs, which may explain why these cells were poised to induce Th1, rather than Th2, immune responses *in vivo*, through counter-regulation of Th2 activation. In contrast, other Th1 polarizing factors, such as CD70[45], although more highly expressed in knock-in DCs, were expressed at very low levels and therefore were unlikely to contribute to the Th1 polarization by the knock-in DCs. Also, IRF4, a transcription factor in DCs that has recently been described to be essential for Th2 development[46], was not different between WT and knock-in cells. However, the GM-CSF-cultured knock-in DCs produced more CXCL10, a chemokine which has been shown to be involved in DC-dependent Th1 responses *in vivo* [39]; CXCL10 production was also dependent on Syk. Th17 priming by the knock-in DCs was normal, likely reflecting the observation that production of IL-6, the key cytokine that drives Th17 differentiation in this model [47], was minimally affected by the knock-in mutation.

Beta2-integrins have been shown to promote tolerance [10], suppress colitis and dermatitis [48,49] and protect against endotoxic shock [11], consistent with regulatory and homeostatic roles of these integrins in immune responses *in vivo*. In addition, autoimmune colitis has been reported in several LAD-I patients, further supporting the notion that the absence of beta2-integrin-mediated function *in vivo* results in unregulated immune responses [50-52]. Beta2-integrins, therefore, play dual roles in the immune system as they influence both the recruitment of leukocytes to sites of inflammation, as well as the regulation of inflammatory responses by restricting signaling in myeloid cells when they interact with their environment. Our report has unraveled novel roles of beta2-integrins in immune cell functions, and has demonstrated that the beta2-integrin-kindlin-3 interaction, in addition to regulating T cell adhesion and homing [1], also plays an unexpected role in restricting GM-CSF-induced Syk signaling, the transcriptional program characteristic of mature migratory DCs, and in regulating Th1/Th2 immune responses. Our results therefore expand on current models of how beta2-integrins regulate immune homeostasis. In addition, the induction of a migratory DC phenotype by targeting beta2-integrins may be of use in the design of more effective dendritic cell-based immunotherapies.

Materials and Methods

Mice

Mice were maintained in the Wellcome Trust Biocentre at the University of Dundee, the School of Biological Sciences, University of Edinburgh, or the University of Helsinki, Finland, in compliance with UK Home Office Animals (Scientific Procedures) Act 1986 and the Finnish Act on Animal Experimentation (62/2006), respectively. Animal experiments were approved by the Home office (UK) and the national Animal Experiment Board (Finland), respectively. We have previously generated the novel *Itgb2* knock-in mice [1]. C57Bl/6 mice were obtained from Charles River and beta2 null mice from JAX (catalogue number 003329). Age- and sex-matched male or female mice aged 8-12 weeks were used in experiments.

Leukocyte isolation and culture

DCs were generated by culturing bone marrow for 10 days in 10ng/ml GM-CSF (Peprotech), with feeding on days 3, 6, and 8. Alternatively, bone marrow cells were cultured in 200ng/ml Flt3 ligand (ImmunoTools) for 8 days, with feeding on day 4. Macrophages were generated by culture of bone marrow cells in M-CSF (Peprotech) for 7 days, with feeding on day 3. Non-treated tissue culture plates were used for all cultures. Neutrophils were isolated from bone marrow by negative selection using microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Neutrophil survival *in vitro* in GM-CSF was determined by duplicate Trypan blue counts after 44hrs of culture.

Reagents

Syk inhibitor II (Calbiochem, CA, USA) and p38 inhibitor (SB203580, Cell Signaling) were used at 10μM. Cytochalasin D (Calbiochem, CA, USA) was used at 10μg/ml. Bafilomycin (Sigma-Aldrich, MO, USA) was used at 12.5nM. IL-3 (R&D systems) was used at the concentrations indicated in the figure. Integrin ligands ICAM-1 (R&D systems) and Fibronectin (Calbiochem) were coated onto cell culture plates at 3μg/ml and 5μg/ml, respectively, by incubation at 4℃ overnight.

Static adhesion assays

ICAM-1 (6µg/ml, R&D Systems, MN, USA) or iC3b (1µg/ml, R&D Systems, MN, USA) were coated in duplicate/triplicate onto flat-bottomed 96-well Maxisorp plates (Nunc) overnight at 4℃. Wells were blocked with 3% human serum albumin for 1hr 15min at 37℃. DC were resuspended at $2x10^6$ cells/ml in binding medium (RPMI plus 0.1% bovine serum albumin, 40mM Hepes and 2mM $MgCl_2$) and stimulated, where indicated, with 200nM PdBu (Sigma-Aldrich, MO, USA). Cells were allowed to adhere for 20min before gentle washing and detection as in [53].

Priming assays

DCs were activated with 100ng/ml LPS (Sigma-Aldrich, MO, USA) overnight and loaded with 1µM ovalbumin peptide 323-339 (AnaSpec, CA, USA) for 90min. After washing, DCs were cultured with purified CD4 OT-II T cells at a ratio of 1:10.

Moloney murine leukaemia virus H19env peptide 123-141 was emulsified in Complete Freund's Adjuvant (CFA, Sigma-Aldrich, MO, USA) using a sonicator. Mice were immunized with a total of 100µg peptide/CFA subcutaneously into the hind legs. Alternatively, DCs were activated with 100ng/ml LPS (Sigma-Aldrich, MO, USA) overnight and loaded with 50µg/ml H19env peptide for 90min. A total of 1-$2x10^6$ LPS-activated peptide-loaded DCs were injected intravenously into mice.

DCs were loaded with 25µg/ml Schistosomal egg antigen (SEA, in-house) or 10µg/ml heat-killed *Propionibacterium acnes* (in-house) overnight. A total of $5x10^5$ cells were injected into the feet of recipient C57Bl/6 mice. 7d post-immunization, popliteal (draining) lymph nodes were extracted, and cells restimulated with antigens (15µg/ml SEA, 1µg/ml heat-killed *P. acnes*, plate-bound anti-CD3 (clone 2C11)) *in vitro* for 72hr. Supernatants were taken and cytokine production analyzed by ELISA using paired antibody kits (ebioscience) according to standard protocols.

Antigen uptake assays

For *in vitro* antigen uptake by BMDCs, cells were cultured with 0.1mg/ml OVA-alexa fluor 647 (Life Technologies) for 2.5hrs at either 4℃ or 37℃. To assess *in vivo* antigen uptake by DCs and migration to lymph nodes, mice were shaved, tape stripped 3 times, and 50µg OVA-alexa fluor 647 injected intracutaneously. After 24hrs, draining lymph nodes were removed, and DC antigen uptake analyzed by flow cytometry.

Optical trapping

DC-T cell interaction forces were measured using a custom optical trapping system (Glass et al, manuscript in preparation). The beam from a Ventus IR, 3 W, continuous wave laser (Laser Quantum, UK) at a wavelength of 1064 nm was expanded to just overfill a Spatial Light Modulator (SLM; Boulder Nonlinear Systems, CO, USA). The SLM consisted of 512x512 individually addressable pixels, altering the phase of the light and projecting a hologram onto the back aperture of the microscope objective. Holograms were calculated and displayed on the SLM using the 'Red Tweezers' software [54] (University of Glasgow), providing flexibility to alter the position of a triple-spot optical trap within the field-of-view and adjust the trap positions relative to each other to reflect the individual cell size. The triple-spot laser beam was directed into a Nikon TE2000-U inverted fluorescence microscope (Nikon, U.K.). The optical tweezers were calibrated with the laser beam power using the escape force method [54].

For initial contact force measurement, DCs were seeded into an Ibidi µ-slide VI $^{0.4}$ (Ibidi, Germany) and purified OT-II T cells added. A single T cell was optically trapped, brought into contact with a DC, and the shutter was immediately closed. After 2min, the trapping laser was un-blocked and the power of the beam increased in increments until the T cell was separated from the DC, at which point the force of the trap was taken to be equal to the interaction force. For quantifying DC-T cell interactions after 24hrs, cells were incubated in an Ibidi µ-slide 8 well before cell pairs were identified. Measurements were taken as described above.

Flow cytometry and tetramer staining

For *ex vivo* analysis of DCs in lymphoid tissues, organs were digested in 1mg/ml collagenase type II (Life Technologies) with 2mM EDTA for 20-30mins at room temperature with agitation. Tetramer staining was performed in 96-well round-bottom plates. PE-labeled MHC class II tetramers (NIH tetramer core facility, USA) containing either the H19env peptide or an irrelevant peptide control were used at 1/100 dilution in RPMI plus 10% fetal bovine serum, for 3hr at 37°C. The following conjugated antibodies were used (all ebioscience, unless otherwise stated): CD18 (C71/16), CD11a (2D7), CD11b (M1/70), CD11c (HL3), CD80 (16-10A1), CD86 (GL1), CD40 (3/23), MHC class II (M5/114.15.2), CD103 (2E7), CD69 (H1.2F3), CD25 (PC61), CD44 (IM7), CD3 (145-2C11), CD4 (RM4-5), CD8a (53-6.7), CD19 (1D3), B220 (RA3-6B2), F4-80 (BM8), Gr-1 (RB6-8C5), CCR7 (4B12),

GM-CSF receptor beta-common chain (K-17, Santa Cruz Biotech). All FITC-conjugated antibodies were used at 1:100 dilution, and all other antibodies were used at 1:200 dilution. Fc block (clone 2.4G2, BD Biosciences, 1:100 dilution) was included in all stains. Propidium iodide or DAPI (Sigma) were added immediately before acquisition on an LSR Fortessa flow cytometer (Becton Dickinson). Data was analyzed using FlowJo software (TreeStar, OR, USA).

Immunoblotting

DCs were lysed in 1% Tx-100, 150 mM NaCl, 50 mM Tris pH 7.4, 10 mM ethylenediamine tetraacetic acid (EDTA), 50 mM NaF, and 1 mM sodium orthovanadate, containing protease inhibitor cocktail (Roche). Alternatively, DCs were fractionated into subcellular fractions using a subcellular protein fraction kit (Thermo Scientific, NH, USA) according to the manufacturer's instructions. Primary antibodies against the following proteins were used (Cell Signaling, unless otherwise stated): p-JAK2, p-STAT5, p-Syk, p-p38, p-Erk, p-Akt, Akt, Src, Syk, beta-actin, beta2-integrin (Abcam, MA, USA), GM-CSF receptor beta-common chain (Santa Cruz Biotechnology, CA, USA), kindlin-3 (Sigma-Aldrich, MO, USA), talin, CD9 (BD Biosciences). After incubation in horseradish peroxidase–conjugated secondary antibodies, blots were developed by standard chemiluminescence techniques. All uncropped blots are shown in Supplementary Figure S7.

RNA preparation and Next-generation sequencing

RNA was prepared from DCs cultured from 3 WT and 3 knock-in mice using the NucleoSpin RNA II kit (Macherey-Nagel). RNA samples were analyzed by the Finnish Microarray and Sequencing Centre (Turku, Finland) after the quality of RNA was checked using the Bioanalyzer (Agilent). Sequencing of samples was performed on the Illumina HiSeq2500 using 1 x 50 bp runs on single-end libraries.

Selection of differentially expressed genes

All Sequencing data in the fastq format was analyzed using the Chipster General User Interface [55]. Sequences were trimmed at the 5'end (minimum score 20) and filtered with a maximum length of 50 bp using PRINSEQ [56]. Reads were aligned using TopHat2 [57] onto the Mouse genome (mm10) for each sample (total genes 37315) and the aligned reads per gene were counted using HTseq. Differential expression analysis was performed by negative binomial distribution using edgeR [58] and

DESeq [59] software. Common genes chosen by both these software packages were chosen and those genes which had an expression level of less than 10 RPKM (Reads per kilobase per million reads) in at least one of the up-regulated samples were discarded. This left a total of 855 genes expressed higher in WT DCs and 862 genes expressed higher in knock-in DCs.

Comparison with resident and migratory DCs

Differentially expressed genes from migratory and resident DCs identified from a previous microarray study [29] were downloaded (Supplementary dataset S3). Genes which were represented more than once through multiple Microarray probes were combined and genes without an ENSMUS number were removed. This left a total of 249 genes up-regulated in migratory DCs and 93 genes up-regulated in resident DCs. The identities of these genes were then compared with the list of differentially expressed genes from our study.

Statistical analysis

The Student's 2-tailed *t*-test, Mann-Whitney test, ANOVA test (Graphpad Prism, CA, USA), and Hypergeometric test (GeneProf) were used to calculate statistical significance. All p-values are shown as <0.05 *, <0.01 **, <0.005 ***.

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Author contributions: V.L.M contributed to planning the study, performed experiments and analyzed data. M.J.J, K.G., P.C., D.G.G., H.S.L., T.S., C.G-B. performed experiments and analyzed data or helped with experiments. C.W. provided vital materials for the study. O.R.M. planned and performed experiments and analyzed data. A.S.M. planned and supervised some experiments. S.C.F. planned and supervised the study, performed experiments and analyzed data. V.L.M and S.F. wrote the paper. All other authors read and commented on the manuscript.

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Figure Legends

Figure 1: The integrin-kindlin link in DCs restricts T cell activation. WT and knock-in (KI) mice were immunized subcutaneously with H19env peptide emulsified in CFA, and the T cell response in the spleen and inguinal (draining) lymph nodes analyzed using MHC class II tetramers 9d later. Each data point represents an individual mouse, with mean displayed, pooled from 2 independent experiments, with 5 mice per group per experiment. Control groups received no immunization. (B) WT or KI resting peptide-loaded BMDCs were cultured with CD4 OT-II T cells *in vitro* at a ratio of 1:10. T cell expression of CD69 (on day 1), CD25 (on day 2) and CD44 (on day 4) was analyzed. Solid line – WT DCs, dashed line – KI DCs. Data is representative of N=4. (C) WT or KI BMDCs were pulsed with 1mg/ml OVA or left un-pulsed before addition of OVA-specific OT-II T cells. The force required to separate DCs and T cells after 2mins or 24hrs of contact was determined. Each data point represents a single cell, with cells taken from 2 mice, and median indicated. p-values were calculated using the Mann-Whitney test. (D) BMDC adhesion to ICAM-1 and iC3b was measured by static adhesion assay. N=4-7. Error bars indicate SEM. (E) BMDCs were imaged after 10d of culture in GM-CSF. Images are representative of N=5. Scale bar is 100μm. (F) WT or KI LPS-activated, peptide-loaded BMDCs were adoptively transferred into WT recipient mice intravenously, and the T cell response in the spleen measured 7d later using MHC class II tetramers. Each data point indicates an individual mouse, with mean displayed. The control group received no adoptive transfer. Data is pooled from 2 independent experiments, with 5 mice per group in each experiment. In (A), (D) and (F) significance values were calculated using the Student's *t*-test. All p-values are shown as <0.05 *, <0.01 **, <0.005 ***.

Figure 2: Beta2-integrin knock-in DCs have elevated responsiveness to GM-CSF. (A) Expression of CD80, CD86, CD40 and MHC class II by WT (solid line) and KI (dashed line) BMDCs after 24hrs stimulation with 10ng/ml GM-CSF or 100ng/ml LPS. Histograms are representative of N=4. Shaded line – isotype control. (B) Expression of activation markers by WT and KI BMDCs following 24hr stimulation with GM-CSF or LPS. N=4. (C) Cytokine production by WT and KI DCs was measured after 24hr stimulation with GM-CSF or LPS. N=3-5. ND = not detectable. (D) Surface expression of GM-CSF receptor by WT and KI BMDCs, showing representative histogram (WT solid line; KI dashed

line; isotype control shaded line) and pooled data N=3 (dashed line represents isotype control). (E) WT and KI BMDC were starved of GM-CSF for 60 min and then stimulated for the indicated times with 10ng/ml GM-CSF, and analyzed for pJAK2 and pSTAT5 by Western blotting. Representative of N=3. (F) WT and KI BMDCs were lysed following 3hr stimulation with fresh addition of 10ng/ml GM-CSF to DC cultures (without GM-CSF starvation), and analyzed for phosphorylated (p-) signaling molecules or Akt (loading control) by Western blotting. Data shows N=2, and is representative of N=4. (G) WT and KI DCs were stimulated with 100ng/ml LPS for up to 60min before lysis. Signaling molecules were analyzed by Western blotting. Data represents N=3. All error bars show SEM and significance values were calculated using the Student's *t*-test. All p-values are shown as <0.05 *, <0.01 **, <0.005 ***.

Figure 3: Beta2-integrin knock-in DCs have a mature migratory phenotype *in vitro*. (A) Heat map of transcripts identified by RNAseq which show at least 2-fold differences in expression between WT and KI BMDCs. N=3. (B) Expression of biologically relevant genes in WT and KI BMDCs identified by RNAseq. N=3. (C) WT and KI BMDCs were incubated with 0.1mg/ml of OVA-alexa fluor 647 for 2.5hrs at either 4℃ (4deg) or 37℃ (37deg). Antigen uptake was then measured by flow cytometry. N=7. (D) CCR7 expression by WT and KI BMDCs was determined by flow cytometry, with representative histogram (WT solid line; KI dashed line; isotype control shaded line) and pooled data from N=3. (E) Venn diagram showing numbers and overlap of the gene expression profiles of *in vivo* migratory DCs [29] and KI GM-CSF-cultured BMDCs. Significance values in (C-D) were calculated using the Student's *t*-test, and the p-value in (E) was calculated by Hypergeometric test. All p-values are shown as <0.05 *, <0.01 **, <0.005 ***.

Figure 4: Beta2-integrin knock-in DCs have a mature migratory phenotype *in vivo*. (A) Numbers of CD11c+ MHC class II+ DCs were quantified in lymphoid tissues of WT and KI mice by flow cytometry. N=4. (B) Resident and migratory DCs were quantified in WT and KI spleen and peripheral lymph nodes. 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+. N=5. (C) Percentages of CD19+ B cells and CD3+ T cells in the spleens and lymph nodes of CFA immunized mice 9d post-immunization. N=5. (D) Percentages of CD11c+ MHC

class II+ DCs in lymph nodes of naïve and CFA immunized mice 24hrs post-immunization. N=3. (E) Expression of CD86 and CD40 on total splenic DCs was examined in resting and CFA-immunized WT and KI mice 24hrs post-immunization. N=3. (F) WT and KI mice received OVA-alexa fluor 647 intracutaneously and 24hrs later the draining lymph nodes were analyzed for OVA+ DCs by flow cytometry. N=5. All error bars show SEM and significance values were calculated using the Student's *t*-test. All p-values are shown as <0.05 *, <0.01 **, <0.005 ***.

Figure 5: Beta2-integrin-mediated ligand binding regulates DC maturation. (A) WT DCs were matured for 24h by LPS-stimulation and assessed for adhesion to ICAM-1 and fibronectin. N=3. (B) WT and beta2 null DC lysates were analyzed for p-Syk, p-Akt and Src (loading control) by Western blotting after 3hr GM-CSF stimulation. Shows N=2, representative of N=3. (C) Expression of CD80, CD86, CD40 and MHC class II by WT (solid line) and beta2 null (dashed line) DCs. Data is representative of N=4 mice. Shaded line – isotype control. (D) Cytokine production by WT, KI and beta2 null (KO) DCs was measured after 24hr stimulation with 10ng/ml GM-CSF or 100ng/ml LPS. N=3. ND = not detectable. (E) WT DCs were adhered on plates or detached and placed in tubes and stimulated for 3h with GM-CSF and analyzed for GM-CSF receptor signaling by Western blotting. Shows N=2, representative of N=3. (F) WT DCs were adhered on plates or detached in tubes for 3hr before being stimulated with GM-CSF for a further 24hr. Expression of CD80, CD86, CD40 and MHC class II was then measured using flow cytometry. N=6. (G) IL-12p40 production by adhered and detached WT DCs in response to GM-CSF, and IL-10 production following LPS stimulation. N=6. (H-I) WT and KI BMDCs were placed on ICAM-1, fibronectin (Fn), non-treated petri dishes or tissue culture-treated plates (TC) for 3hrs, and then stimulated with GM-CSF for a further 24hrs. CD86 expression (H) and IL-12p40 production (I) were then measured. N=3. All error bars show SEM and significance values were calculated using the Student's *t*-test. All p-values are shown as <0.05 *, <0.01 **, <0.005 ***.

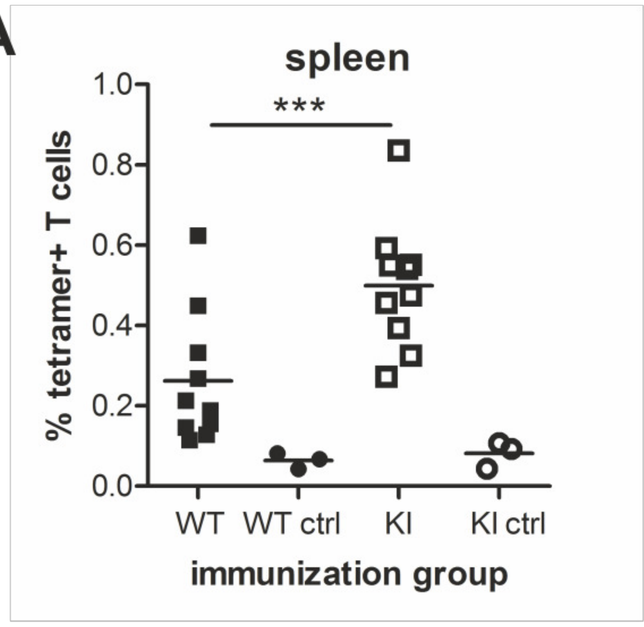
Figure 6: Integrin-kindlin-actin links regulate DC maturation. (A) DC lysates were separated into subcellular fractions using a commercial kit (Thermo Scientific), as described in the methods section, and then analyzed by Western blotting. C=cytoplasmic, M=membrane, CS=cytoskeleton. Shows N=1 and is representative of N=4. (B) p-Syk, p-Erk and Src (loading control) levels in lysates from WT DCs

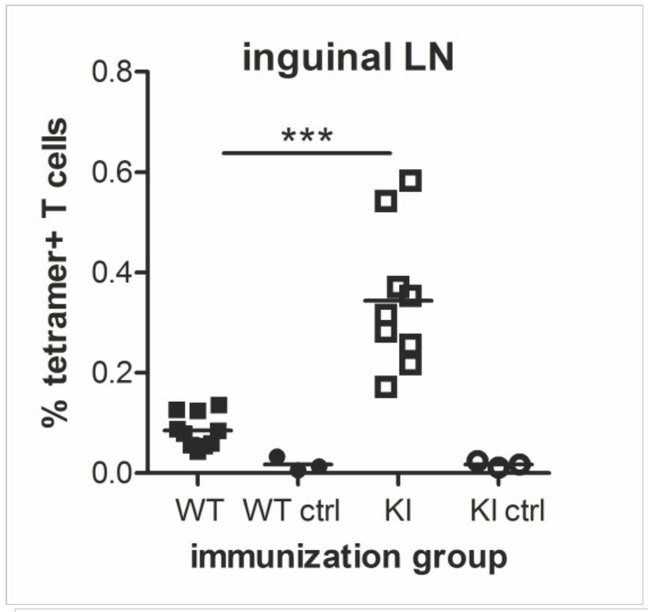
stimulated with GM-CSF, either untreated or treated with 10µg/ml cytochalasin D (CytoD). N=2 is shown, and is representative of N=3. (C) WT DC expression of CD86 and CD40 in plates and tubes, with treatment with CytoD. N=4. (D) IL-12p40 production by WT DCs in plates and tubes, with CytoD treatment, following GM-CSF stimulation. N=4. Error bars show SEM. The Student's *t*-test was used to calculate p-values.

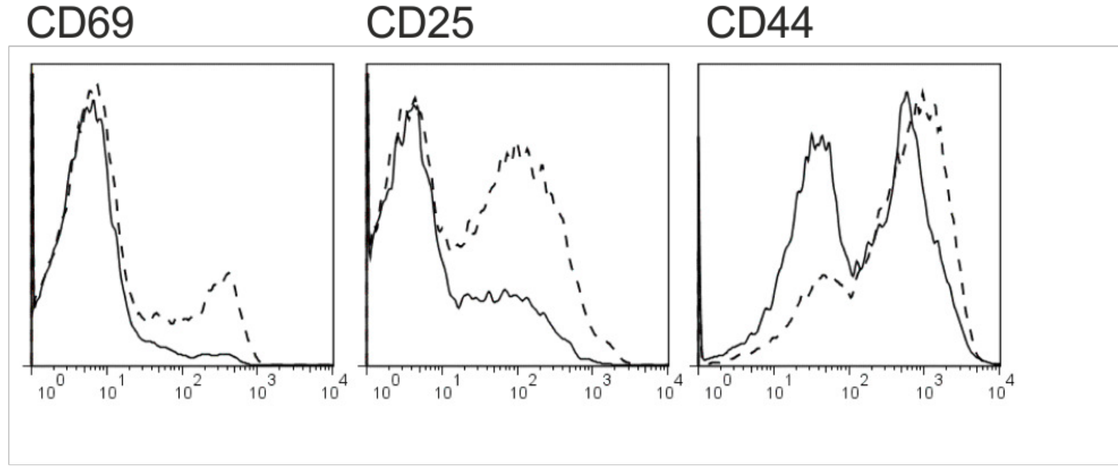
Figure 7: Syk is partly responsible for the mature DC phenotype. (A-B) WT and KI BMDCs were put in plates or tubes and pre-treated with Syk and p38 inhibitors for 30mins before the addition of 10ng/ml GM-CSF or 100ng/ml LPS for 24hrs. Expression of CD80, CD86, CD40 and MHC class II was then analyzed by flow cytometry, N=4-6 (A). Levels of IL-12p40 and IL-10 were measured in culture supernatants after GM-CSF (for IL-12p40) or LPS (for IL-10) stimulation. N=4-6. (C) WT, KI and beta2 null (KO) DCs were pre-treated with Syk inhibitor II for 30mins, and then stimulated with GM-CSF (for IL-12p40) or LPS (for IL-10) for 24hrs. N=3. ND = not detectable. In all cases error bars show SEM. The Student's *t*-test was used to calculate p-values. All p-values are shown as <0.05 *, <0.01 **, <0.005 ***.

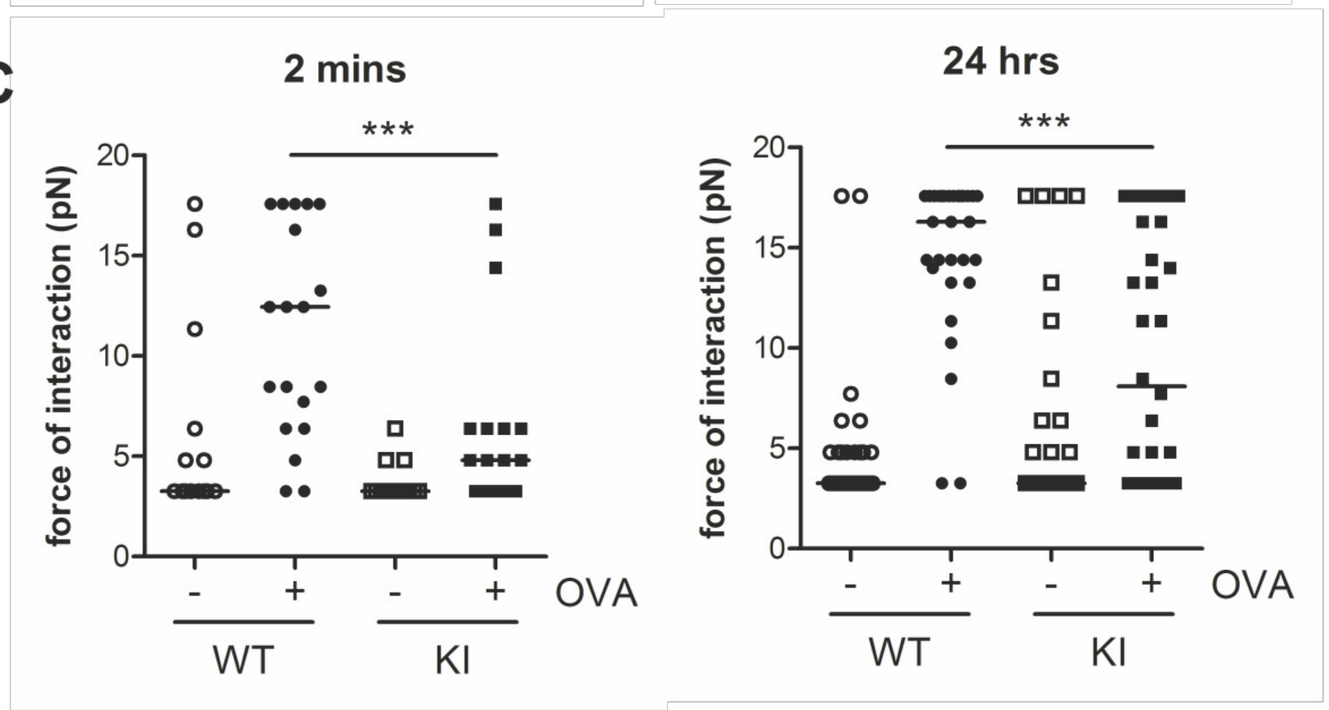
Figure 8: Beta2-integrin knock-in DCs induce elevated Th1 responses. (A) WT or KI DCs were loaded with SEA or heat-killed *P. acnes* prior to being transferred into WT recipient mice subcutaneously into the foot. On day 7, the popliteal (draining) lymph nodes were removed, and the T cell response analyzed by measuring cytokine production following *in vitro* restimulation with antigen. Presented data is from one experiment, and is representative of 2 independent experiments, with 5 mice per group per experiment. (B) CXCL10 production was measured after 24hrs stimulation of WT and KI DCs in the presence or absence of the Syk inhibitor. N=4. In all cases, error bars indicate SEM. The Student's *t*-test was used to calculate p-values. NS = not significant. ND = not detectable. All p-values are shown as <0.05 *, <0.01 **, <0.005 ***.

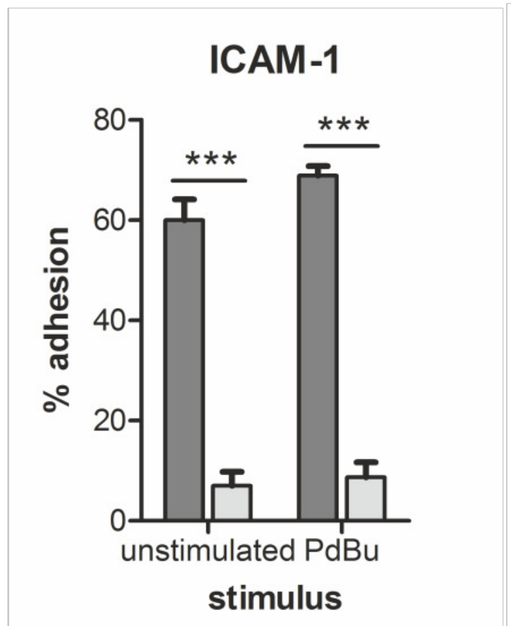
Figure 1

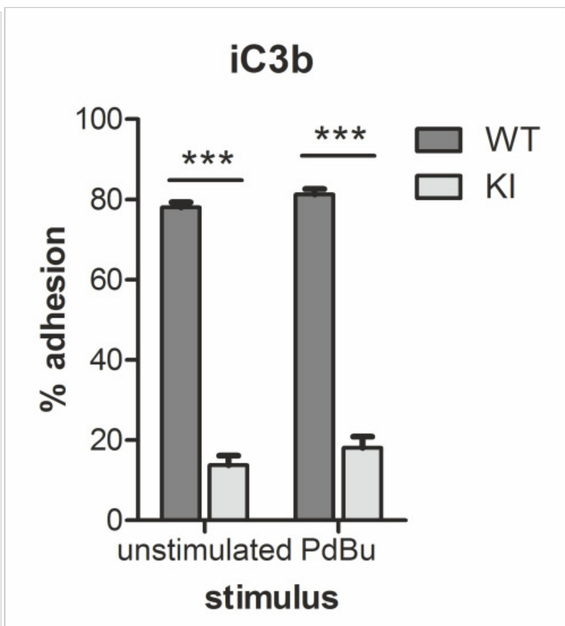

A
spleen
% tetramer+ T cells
1.0
0.8
0.6
0.4
0.2
0.0
***
WT
WT ctrl
KI
KI ctrl
immunization group

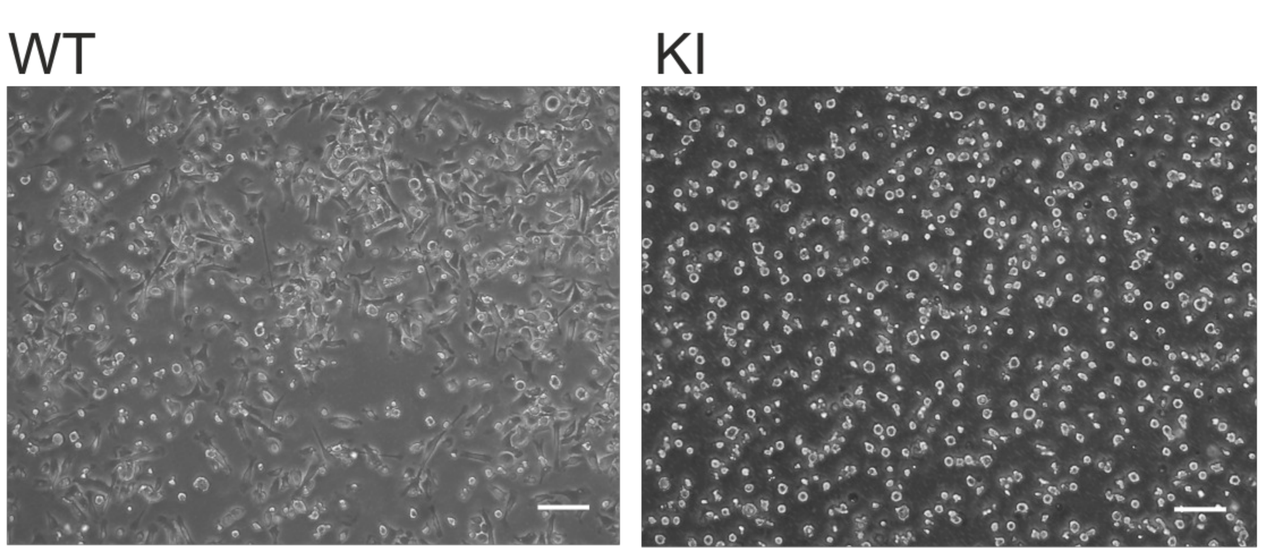

inguinal LN
% tetramer+ T cells
0.8
0.6
0.4
0.2
0.0
***
WT
WT ctrl
KI
KI ctrl
immunization group


B
CD69
CD25
CD44


C
2 mins
24 hrs
***
force of interaction (pN)
20
15
10
5
0
-
+
OVA
WT
KI


D
ICAM-1
% adhesion
80
60
40
20
0
***
unstimulated
PdBu
stimulus


iC3b
% adhesion
100
80
60
40
20
0
***
WT
KI
unstimulated
PdBu
stimulus


E
WT
KI

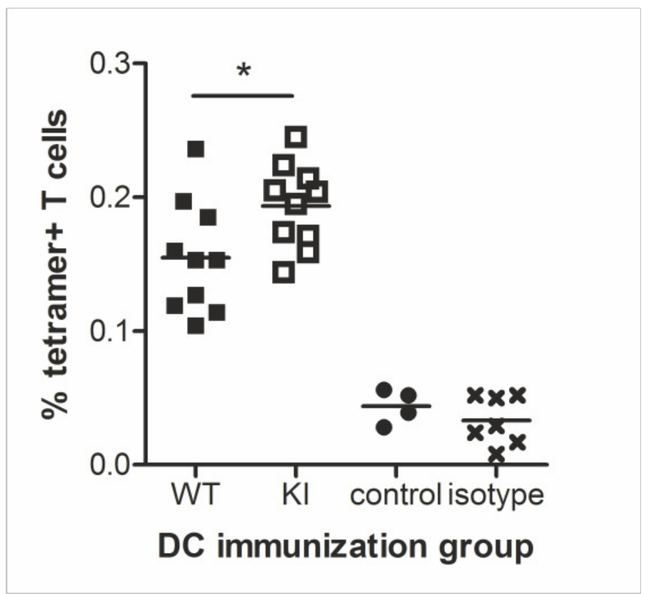

F
% tetramer+ T cells
0.3
0.2
0.1
0.0
*
WT
KI
control
isotype
DC immunization group

Figure 2

A
CD80
CD80
CD80
MHC class II
GM-CSF
LPS
B
MFI CD86
MFI CD80
MFI CD40
MFI MHC class II
GM-CSF
LPS
stimulus
WT
KI
NS
C
IL-12p40 (ng/ml)
IL-10 (ng/ml)
ND
D
GM-CSF R
MFI GM-CSF receptor
-LPS
+LPS
treatment
E
WT KI WT KI WT KI
p-JAK2
p-STAT5
0 0 30 30 60 60 GM-CSF (min)
F
WT1 WT2 KI1 KI2
p-JAK2
p-STAT5
p-Syk
p-p38
p-Erk
p-Akt
Akt
G
WT
KI
p-Syk
p-IKKalpha/beta
p-Akt(Thr308)
p-p38
p-Erk
Akt
0 30 60 0 30 60 LPS (min)

Figure 3

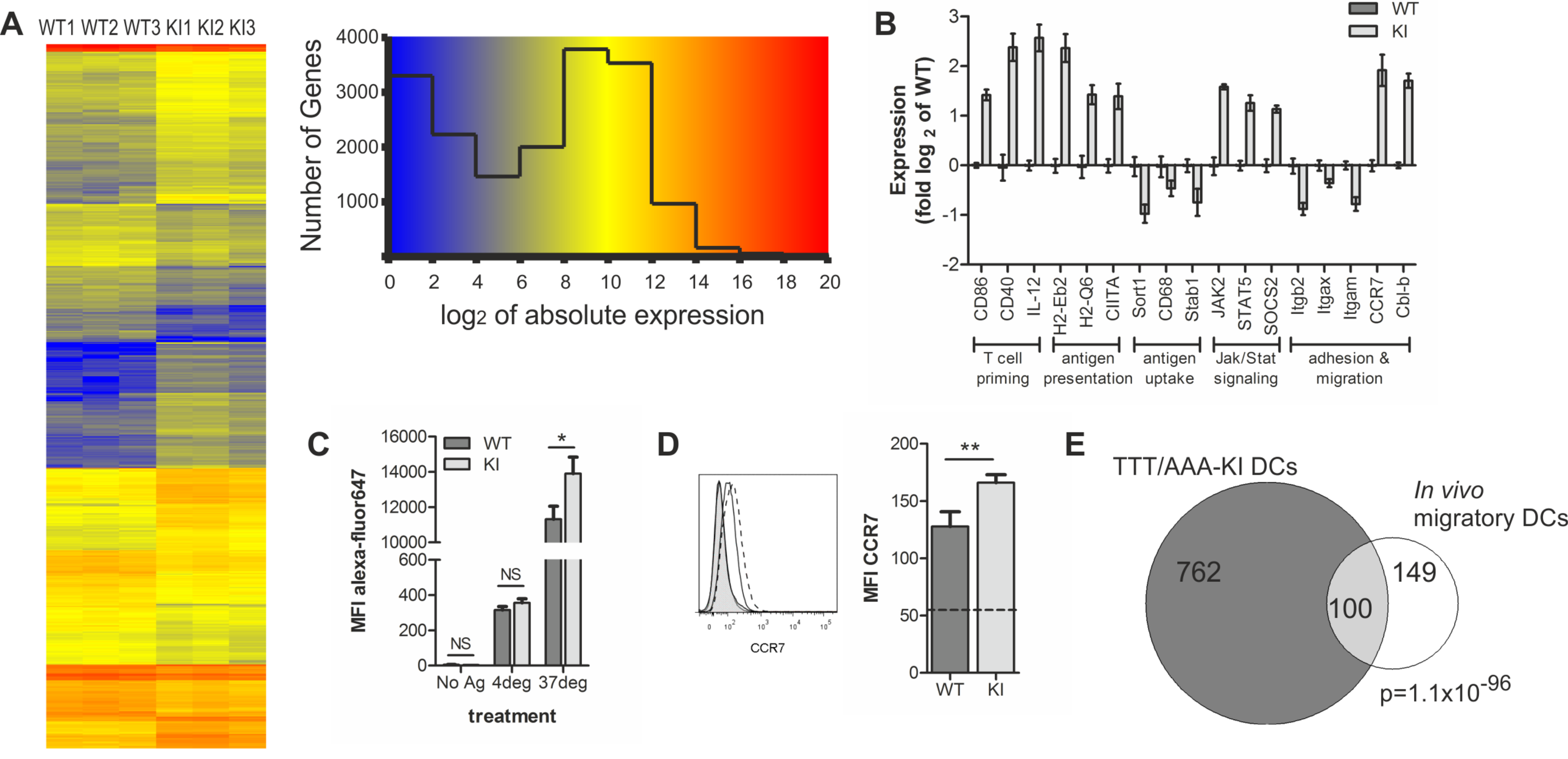

A
WT1 WT2 WT3 KI1 KI2 KI3
Number of Genes
4000
3000
2000
1000
0 2 4 6 8 10 12 14 16 18 20
log2 of absolute expression
B
WT
KI
Expression (fold log 2 of WT)
3
2
1
0
-1
-2
CD86 CD40 IL-12 H2-Eb2 H2-Q6 CIITA Sort1 CD68 Stab1 JAK2 STAT5 SOCS2 Itgb2 Itgax Itgam CCR7 Cbl-b
T cell priming
antigen presentation
antigen uptake
Jak/Stat signaling
adhesion & migration
C
MFI alexa-fluor647
16000
14000
12000
10000
600
400
200
0
WT
KI
*
NS
NS
No Ag 4deg 37deg
treatment
D
CCR7
MFI CCR7
200
150
100
50
0
**
WT KI
E
TTT/AAA-KI DCs
In vivo migratory DCs
762
100
149
p=1.1x10-96

Figure 4

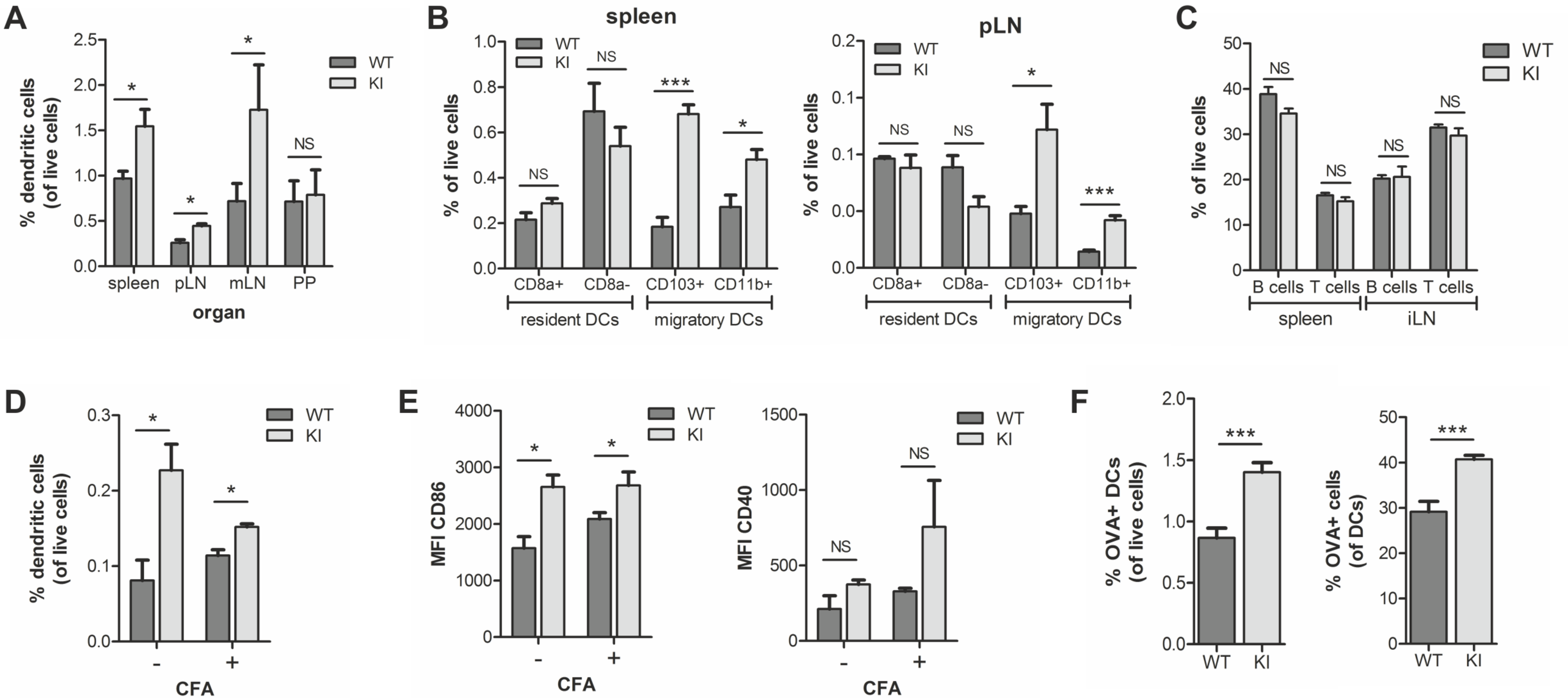
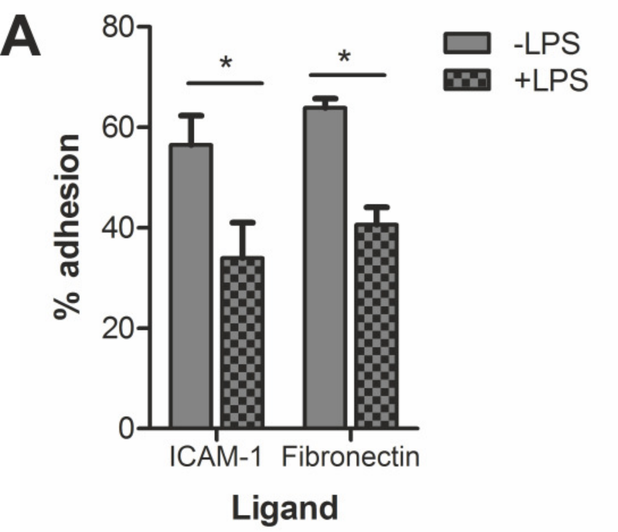
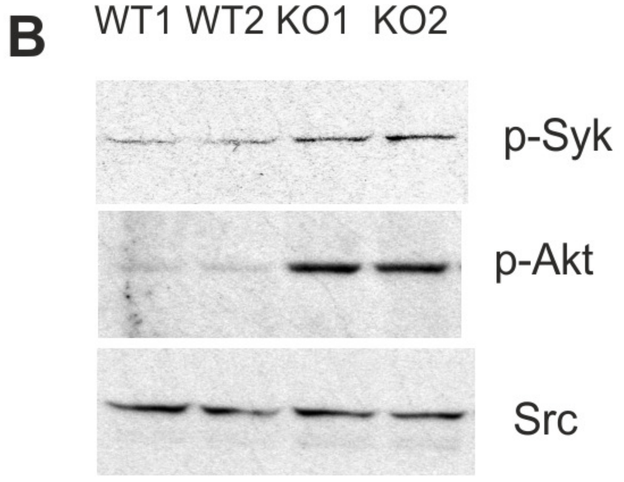
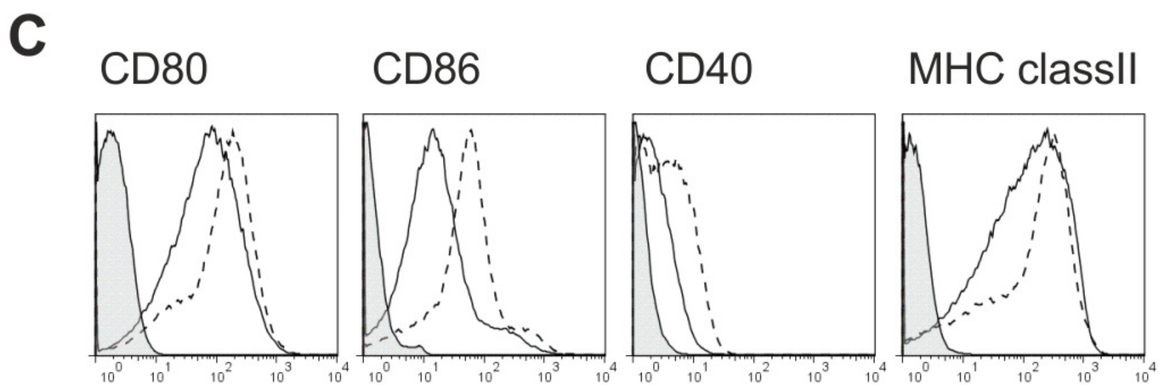
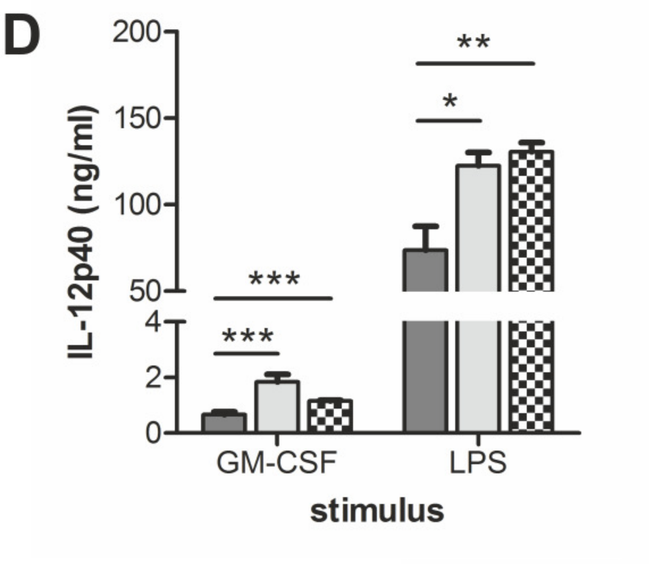

A
% dendritic cells (of live cells)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
NS
WT
KI
spleen
pLN
mLN
PP
organ
B
spleen
% of live cells
1.0
0.8
0.6
0.4
0.2
0.0
WT
KI
NS
NS
***
*
CD8a+
CD8a-
CD103+
CD11b+
resident DCs
migratory DCs
pLN
% of live cells
0.2
0.1
0.1
0.0
0.0
WT
KI
NS
NS
*
***
CD8a+
CD8a-
CD103+
CD11b+
resident DCs
migratory DCs
C
% of live cells
50
40
30
20
10
0
WT
KI
NS
NS
NS
NS
B cells
T cells
B cells
T cells
spleen
iLN
D
% dendritic cells (of live cells)
0.3
0.2
0.1
0.0
*
*
WT
KI
-
+
CFA
E
MFI CD86
4000
3000
2000
1000
0
WT
KI
*
*
-
+
CFA
MFI CD40
1500
1000
500
0
WT
KI
NS
-
+
CFA
F
% OVA+ DCs (of live cells)
2.0
1.5
1.0
0.5
0.0
***
WT
KI
% OVA+ cells (of DCs)
50
40
30
20
10
0
***
WT
KI

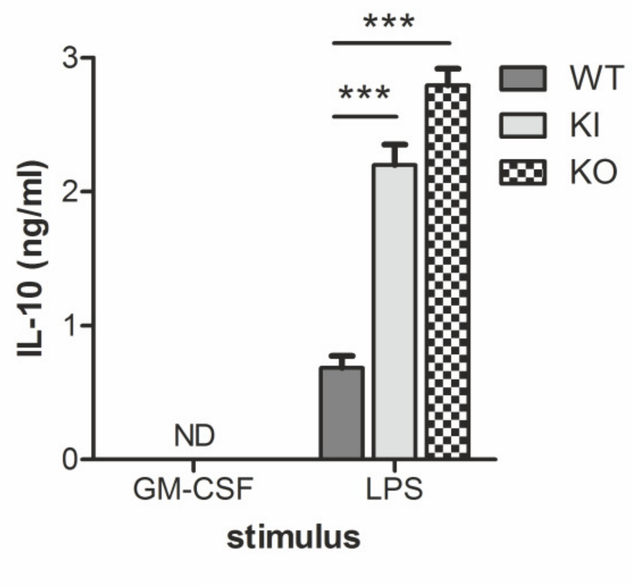
Figure 5

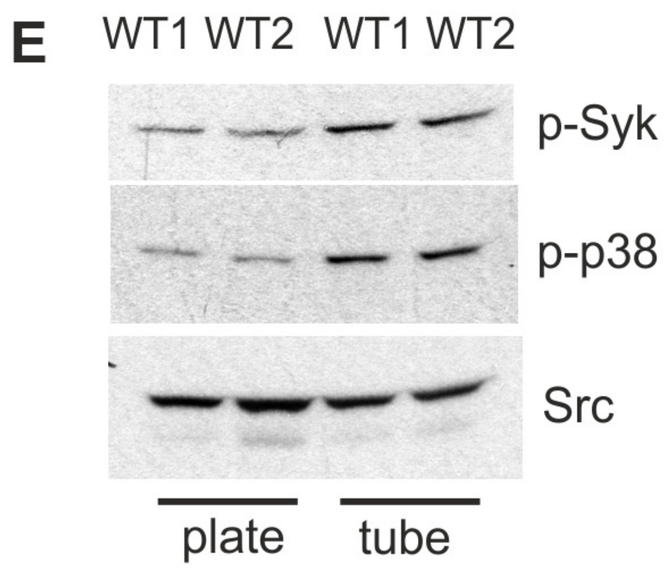

A
80
60
40
20
0
% adhesion
*
*
-LPS
+LPS
ICAM-1
Fibronectin
Ligand

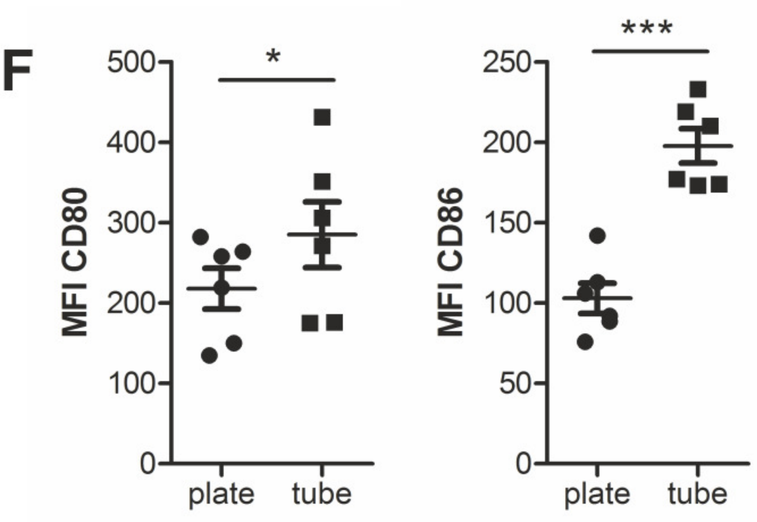

B
WT1 WT2 KO1 KO2
p-Syk
p-Akt
Src

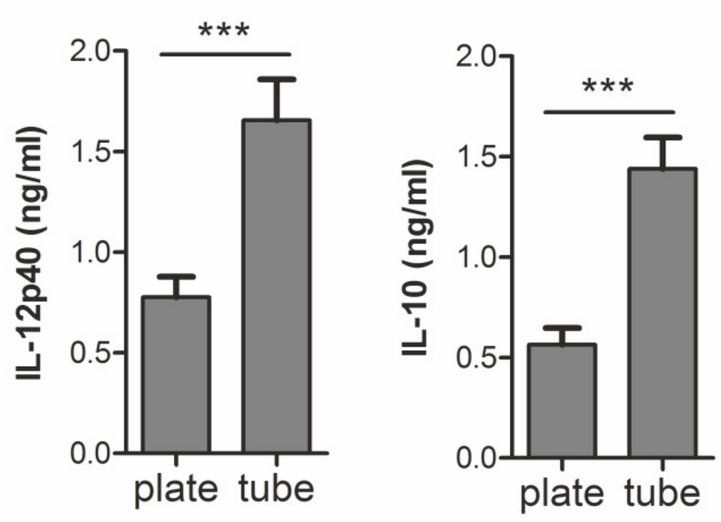

C
CD80
CD86
CD40
MHC classII

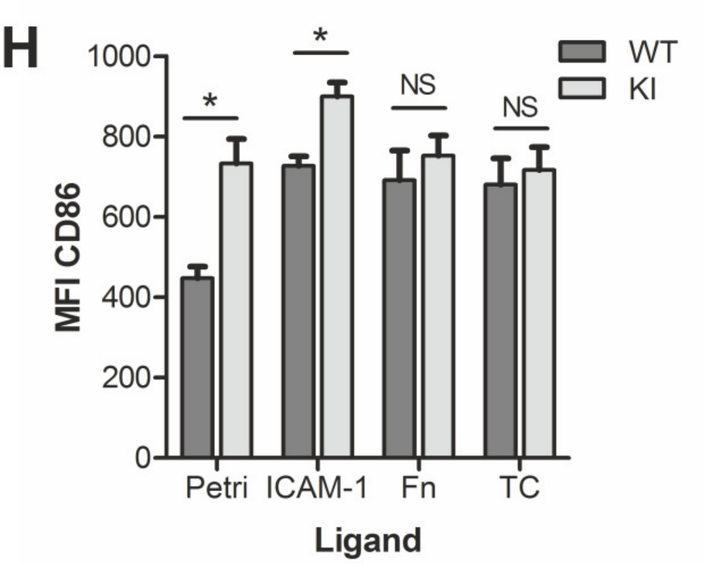

D
200
150
100
50
4
2
0
IL-12p40 (ng/ml)
***
***
**
*
GM-CSF
LPS
stimulus


3
2
1
0
IL-10 (ng/ml)
***
***
ND
WT
KI
KO
GM-CSF
LPS
stimulus


E
WT1 WT2 WT1 WT2
p-Syk
p-p38
Src
plate
tube


F
500
400
300
200
100
0
MFI CD80
*
plate
tube
250
200
150
100
50
0
MFI CD86
***
plate
tube
5
4
3
2
1
0
MFI CD40
*
plate
tube
1500
1000
500
0
MFI MHC class II
**
plate
tube


G
2.0
1.5
1.0
0.5
0.0
IL-12p40 (ng/ml)
***
plate
tube
2.0
1.5
1.0
0.5
0.0
IL-10 (ng/ml)
***
plate
tube


H
1000
800
600
400
200
0
MFI CD86
*
*
NS
NS
WT
KI
Petri
ICAM-1
Fn
TC
Ligand

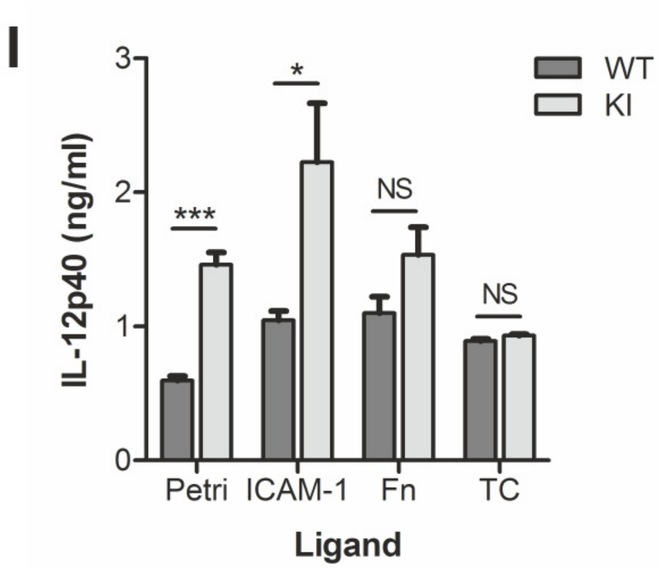
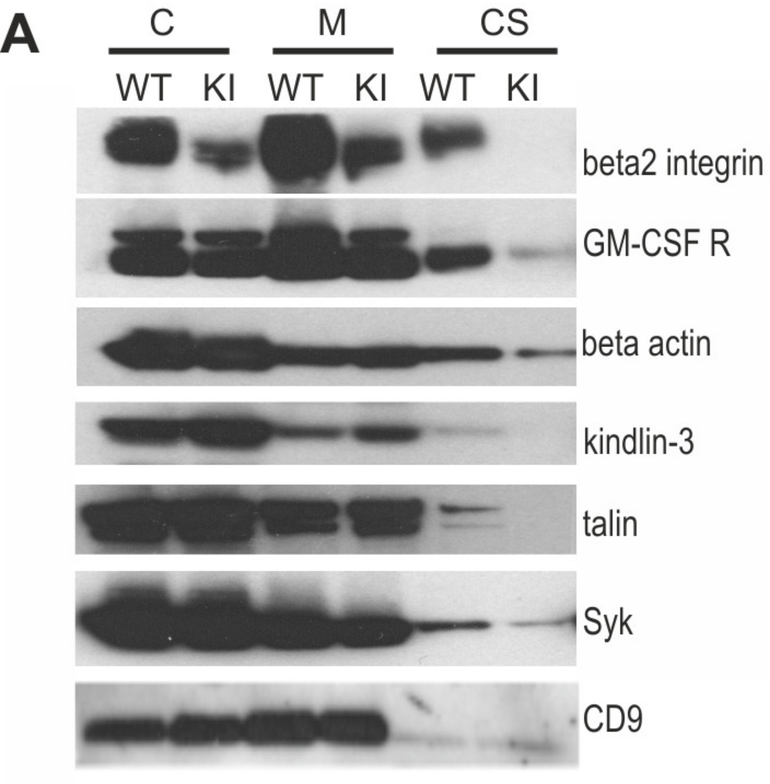
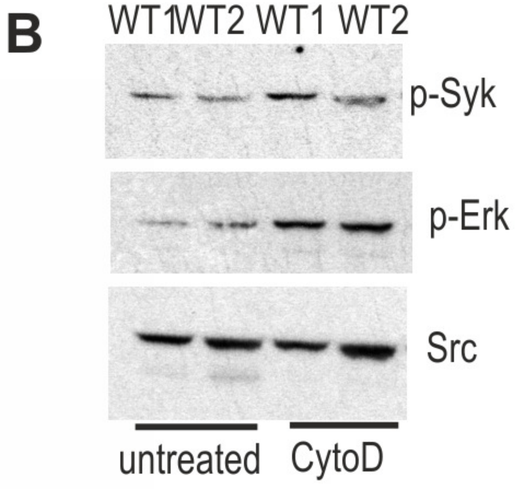
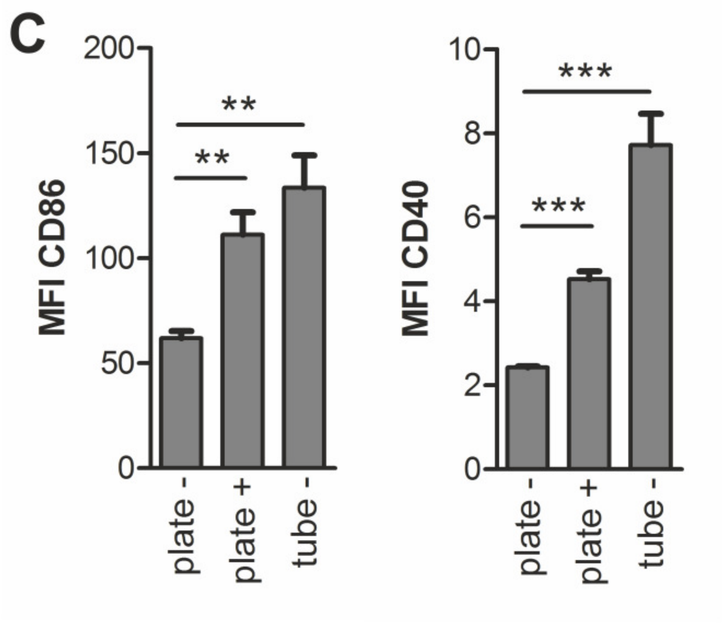

I
3
2
1
0
IL-12p40 (ng/ml)
***
*
NS
NS
WT
KI
Petri
ICAM-1
Fn
TC
Ligand

Figure 6


A
C
M
CS
WT
KI
WT
KI
WT
KI
beta2 integrin
GM-CSF R
beta actin
kindlin-3
talin
Syk
CD9


B
WT1
WT2
WT1
WT2
p-Syk
p-Erk
Src
untreated
CytoD


C
MFI CD86
200
150
100
50
0
**
**
plate -
plate +
tube -
MFI CD40
10
8
6
4
2
0
***
***
plate -
plate +
tube -

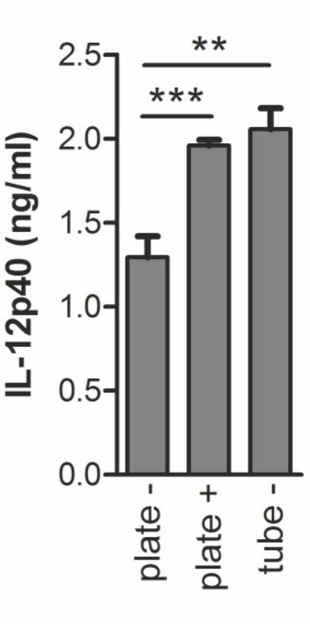

D
IL-12p40 (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
**
***
plate -
plate +
tube -

Figure 7

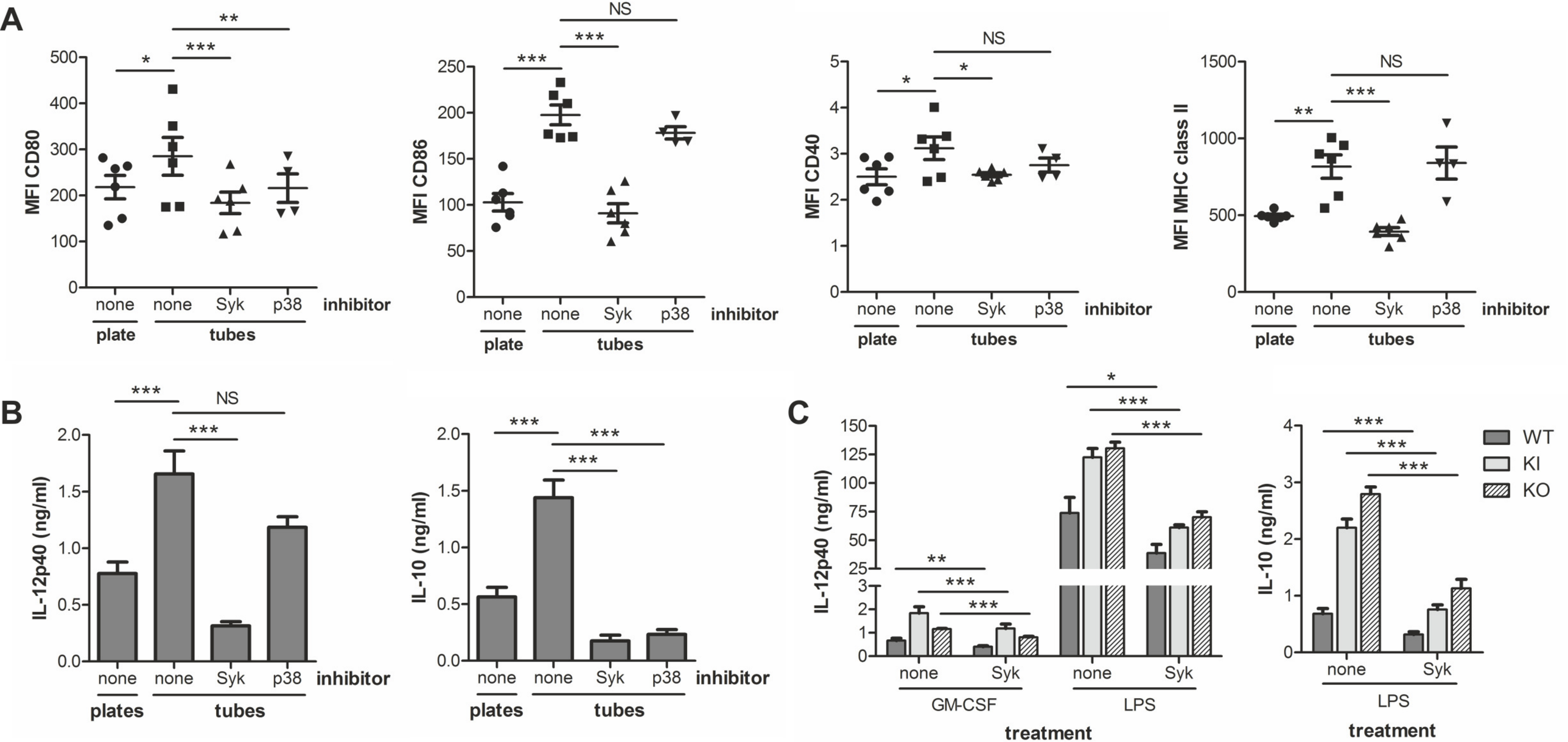
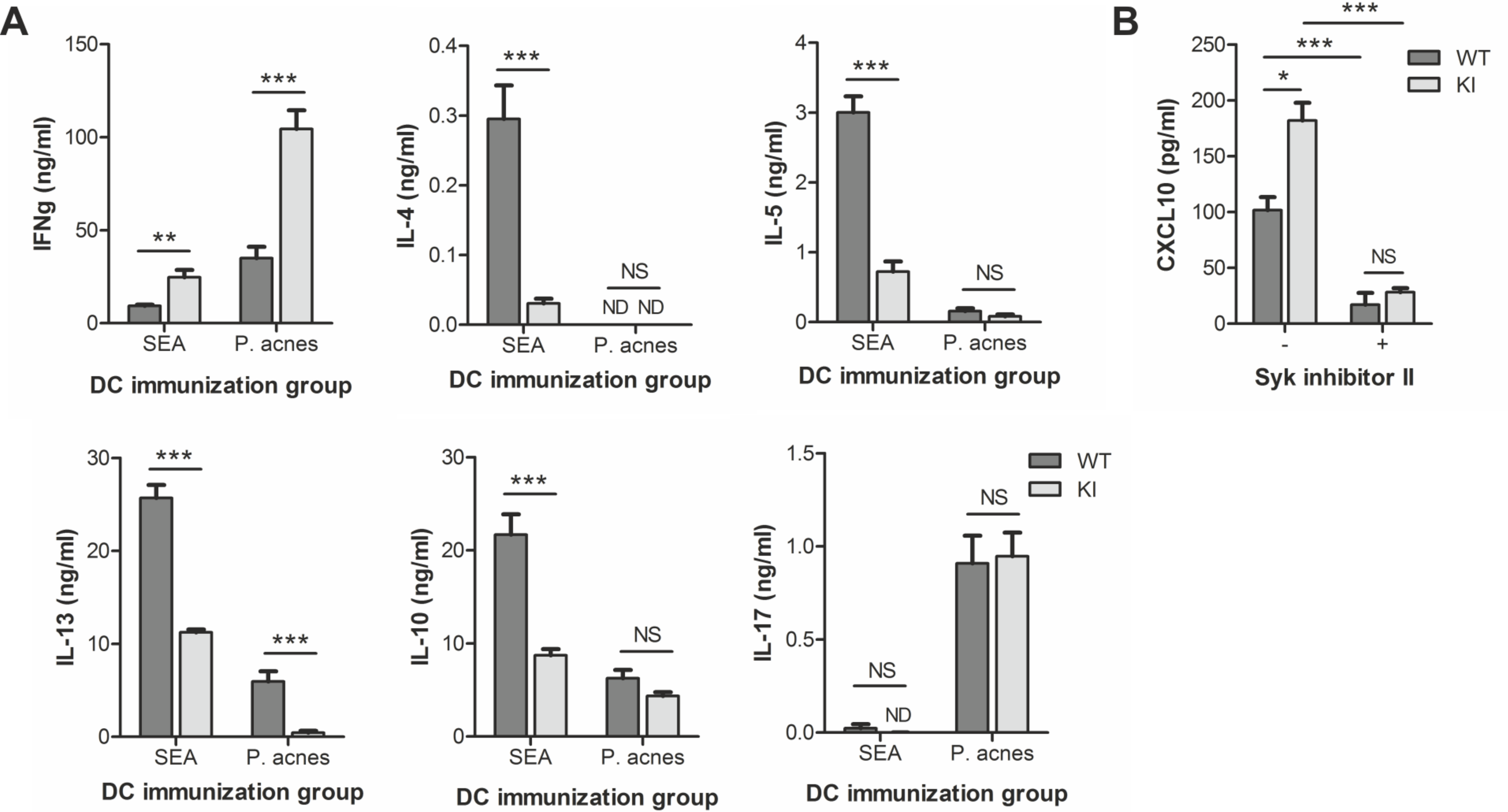

A
MFI CD80
MFI CD86
MFI CD40
MFI MHC class II
none
none
Syk
p38
inhibitor
plate
tubes
NS
B
IL-12p40 (ng/ml)
IL-10 (ng/ml)
plates
tubes
C
WT
KI
KO
GM-CSF
LPS
treatment

Figure 8


A
IFNg (ng/ml)
150
100
50
0
**
***
SEA
P. acnes
DC immunization group
IL-4 (ng/ml)
0.4
0.3
0.2
0.1
0.0
***
NS
ND ND
SEA
P. acnes
DC immunization group
IL-5 (ng/ml)
4
3
2
1
0
***
NS
SEA
P. acnes
DC immunization group
B
CXCL10 (pg/ml)
250
200
150
100
50
0
***
***
*
NS
WT
KI
-
+
Syk inhibitor II
IL-13 (ng/ml)
30
20
10
0
***
***
SEA
P. acnes
DC immunization group
IL-10 (ng/ml)
30
20
10
0
***
NS
SEA
P. acnes
DC immunization group
IL-17 (ng/ml)
1.5
1.0
0.5
0.0
NS
NS
ND
WT
KI
SEA
P. acnes
DC immunization group