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The role of glycoprotein 130 in homing

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Thesis

EXPLORING THE ROLE OF GLYCOPROTEIN 130 IN HOMING

by

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ABSTRACT

Coordinated lymphocyte adhesion and migration is a hallmark of the adaptive immune response in both physiological and pathological conditions. Therefore, understanding the mechanisms underlying lymphocyte trafficking at the molecular level may provide novel targets for the treatment of immune-mediated diseases. The controlled migratory pattern of lymphocytes, commonly referred to as homing, is critically dependent on specialized microvasculature and is facilitated by the expression of adhesion molecules and signaling chemokines. During steady state conditions, homing of immune cells occurs continuously in the lymph nodes due to the constitutive expression of homing molecules, whereas during an inflammatory condition, a reactive up regulation of these adhesion molecules is necessary for immune cell trafficking to take place.

The dynamics of immune cell recruitment, demonstrated by intravital microscopy, showed that lymphocyte adhesion in the target tissue's microvascular bed is mostly restricted to the post-capillary and collecting venules, whereas arterioles and capillaries can not support this interaction. High shear stress exerted by fluid dynamics in the lumen of venules requires intravascular lymphocytes to anchor using receptor molecules that form mechanically stable bonds with counter receptors in the vascular wall (von Andrian & Mackay, 2000). These molecules play a key role in lymphocyte binding and facilitate the directed migration of lymphocytes by functioning as a tissue specific recognition molecule differentially expressed on the surface of lymph node ECs (von Andrian &

Mackay, 2000). Specialized venular ECs found in lymph nodes and Peyer's Patches (PPs) called high endothelial venules (HEVs) constitutively express on their surface a specialized type of this homing molecule called addressins, allowing for the continuous recruitment of lymphocytes during steady state conditions. Elsewhere in the body, ECs must be activated by exposure to inflammatory mediators in order to allow transendothelial migration to the inflamed tissue.

There is strong evidence to show the homing signature of a lymphocyte is dependent on the expression of adhesion molecules and chemokine receptors on the cell surface as well as their ligands expressed by the venular endothelium. Here, we hypothesize that the cytokine signaling receptor subunit glycoprotein 130 (gp130) is a functional requirement for eliciting an effective recruitment of lymphocytes to secondary lymphoid organs during steady states. Glycoprotein 130 is a signaling subunit involved with the interleukin-6 (IL-6) family of cytokines. Previous studies done in the lab have shown that this glycoprotein is over expressed in the venular versus non-venular endothelial cells, indicating a potential role of gp130 in lymphocyte homing during steady state conditions.

This hypothesis was tested by analyzing short term homing assays using donor β -actin-GFP splenocytes, with recipient litter-mate controls and recipient conditional or inducible conditional knockout mouse models that lack gp130 expression on ECs. By comparing the homing abilities of GFP⁺ splenocytes to various secondary lymphoid organs in wild type versus knockout mouse models, we were able to determine that gp130 expression on the endothelial cell compartment does have a role in lymphocyte

homing, demonstrated by impaired homing capabilities evident in only the knockout mice.

Identifying the function of gp130 expressed by venular ECs and its role in lymphocyte recruitment during steady state conditions may lead to a better understanding of the immune system and its complexity during the dynamic maintenance of homeostatic health.

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LIST OF ABBREVIATIONS

APC.....	Antigen Presenting Cell
aurLN	Auricular Lymph Nodes
DC.....	Dendritic Cell
BEC.....	Blood Endothelial Cells
CNTF	Ciliary Neurotrophic Factor
CLC.....	Cardiotrophin-Like Cytokine
cLN	Cervical Lymph Nodes
CT-1	Cardiotrophin-1
DOX.....	Doxycycline
ECs.....	Endothelial Cells
FBS	Fetal Bovine Serum
FDCs	Follicular Dendritic Cells
FRCs	Fibroblastic Reticular cells
GeoMFI.....	Geometric Mean Fluorescent Intensity
Gp130.....	Glycoprotein 130
GFP	Green Fluorescent Protein
Het.....	Heterozygous
HEVs.....	High Endothelial Venules
HUVEC.....	Human Umbilical Venular Endothelial Cells

ICAM-1,2.....	Intercellular Adhesion Molecules 1, 2
IL-6	Interleukin 6
IVM.....	Intravital Microscopy
JAK.....	Janus-Activated Kinase
KO.....	Knockout
LEC.....	Lymphatic Endothelial Cells
LFA-1.....	Lymphocyte Associated Antigen 1
LIF.....	Leukemia Inhibitory Factor
LTi.....	Lymphoid Tissue Inducer Cells
MAPK.....	Mitogen-Activated Protein Kinase
MLNs	Mesenteric Lymph Nodes
MAdCAM-1.....	Mucosal Addressin Cell Adhesion Molecule 1
NPN.....	Neuropoietin
OSM.....	Oncostatin M
PNAd.....	Peripheral Lymph Node Addressin
PLNs	Peripheral Lymph Nodes
PCR.....	Polymerase Chain Reaction
PPs.....	Peyer's Patches
SEM	Standard Error of the Mean
SLOs	Secondary Lymphoid Organs
STAT.....	Signal Transducer and Activator of Transcription

tTA Tetracycline Transactivator protein
Tet-O Tet-operator
VE-Cad VE-Cadherin
WASP Wiskott-Aldrich Syndrome Protein
WT Wild type

INTRODUCTION

Overview of the Immune System

Lymphocytes are a well-characterized type of white blood cells that play a key role in maintaining the health and homeostasis of our bodies. As an important cellular component of the adaptive immune system, these cells have the unique capacity to mount an antigen specific response.

Lymphocytes are generated in the primary lymphoid organs, arising from hematopoietic stem cells in the bone marrow. Before entering circulation, pre-mature lymphocytes are subjected to a selection process and tested for auto reactivity. Once in the periphery, these naïve T cells and B cells navigate through the body in search of their cognate antigen. Following identification of cognate antigen, lymphocytes are activated to undergo a dynamic maturation and differentiation process to enhance their ability to detect those antigens, and in doing so B and T lymphocytes increase their ability to defeat and eliminate pathogens that are the source of the targeted antigens.

Given the vastness of the human body, the challenge then comes with how it is these lymphocytes encounter their cognate antigen. To address this, the immune system has evolved secondary lymphoid organs (SLO) to orchestrate the detection of antigen by lymphocytes and consequently trigger an immune response. SLOs include peripheral lymph nodes (PLNs), mesenteric lymph nodes (MLNs), spleen, Peyer's Patches, appendix and tonsils (Girard & Springer, 1995). SLOs are connected to the parenchyma by the lymphatic system consisting of blind-ending vessels lined by lymphatic endothelial cells (LECs). Afferent lymphatics are found only in lymph nodes and

function to bring in the interstitial fluid from the parenchyma to the draining lymph nodes. Efferent lymphatics exist in all SLOs including the spleen and Peyer's patches and drain the collected fluid out of SLOs. Collectively, this network of lymphatic vessels is responsible for draining interstitial fluids of different tissues through the various SLOs, ultimately ending in the thoracic duct for re-entry into the blood circulation. This fluid called lymph can contain any debris, particulate material, soluble particles including microbes, and antigen presenting cells (APCs).

Not only can free antigen enter SLOs via lymphatic vessels, but specialized antigen presenting cells (APCs) called dendritic cells (DCs), have the capacity to enter lymph nodes via the afferent lymphatics as well (Figure 1). DCs are abundantly present in the SLOs themselves, and are common to areas in direct contact with the outside environment, such as the mucosa, skin, and intestine. Once these peripheral DCs acquire antigen and enter the lymphatics, they drain to proximal lymph nodes where they present antigen on their cell surface for recognition by lymphocytes a process known as antigen presentation. By such interactions with T lymphocytes, these antigen presenting DCs have the critical role of orchestrating the adaptive immune response.

This process of antigen delivery from the periphery increases the efficiency of lymphocytes encountering antigen, ultimately allowing for a more immediate and robust immune response. Unlike DCs, lymphocytes that have not yet recognized their cognate antigen, also known as naïve, do not enter SLOs via afferent lymphatics. Instead, they penetrate specialized postcapillary venular endothelial cells called high endothelial

venules (HEVs) located in the cortex of all lymphoid organs except for the spleen (Girard, Moussion, & Förster, 2012a; von Andrian & Mempel, 2003).

In addition to DCs capturing antigen and draining to SLOs, the immune system has developed another mechanism aiming to efficiently detect antigen by lymphocytes. This design promotes circulating lymphocytes to travel to the SLOs in a non-random fashion and importantly, to remain there when cognate antigen has been detected. The process of directed lymphocyte migration to SLOs at steady state is referred to as homing. It is important to recognize that homing to SLOs serves the purpose of enhancing the availability of cognate antigen for lymphocyte detection. This action is attributable to the sustained chemoattractant gradient produced in the cortex of lymphoid organs by the stromal architecture comprised of fibroblastic reticular cells (FRCs) and follicular dendritic cells (FDCs) that provide the lymph node with distinct chemokines that recruit lymphocytes. In addition, HEVs have been well characterized to constitutively synthesize and present chemokines and surface adhesion proteins involved in recruiting lymphocytes in a process known as the multistep adhesion cascade.

In the case of an immune response, lymphocytes will also be recruited to sites of inflammation following the similar multistep principles, although, this recruitment process is distinguishable in that non-lymphoid organs require the presence of inflammatory signals to mediate venular endothelium lymphocyte trafficking (Girard & Springer, 1995).

Lymphocytes necessitate a dynamic interaction with ECs in order to adhere to the vessel wall and subsequently migrate through the endothelial barrier to enter into the

tissue, a process known as diapedesis. Prior to this transmigration, the lymphocyte must first overcome the high shear stress exerted by the luminal fluid. This process has been well studied and characterized by a series of steps that make up the multistep adhesion cascade.

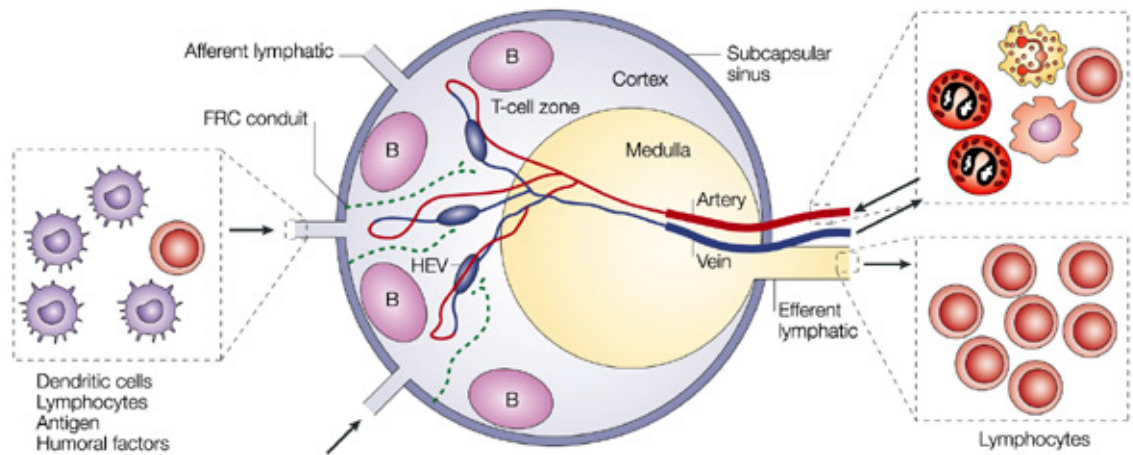
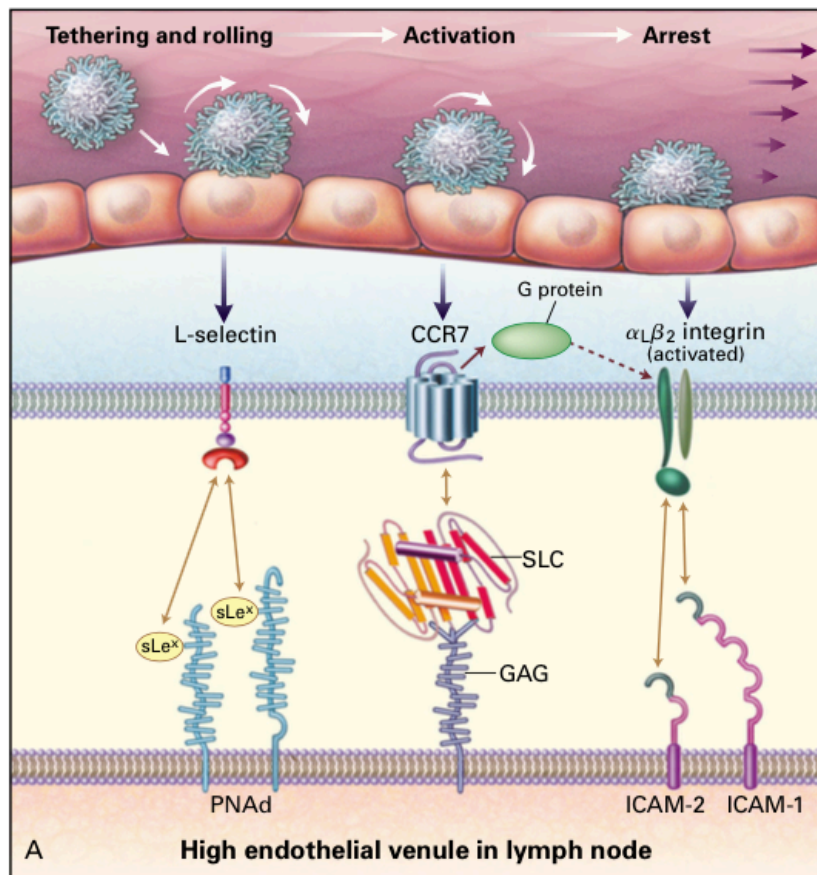


Figure 1. Schematic of lymph node structure. Diagramed is a lymph node depicting the B cell zones (B), T-cell zones, high endothelial venules (HEVs), fibroblastic reticular cell (FRC) conduits and other constituents involved in the immune response. Image from Miyasaka & Tanaka, (2004).

Multistep Adhesion Cascade

During steady-state, naïve T cells display cell surface receptor that allow for their homing to lymph nodes. Once immune cells identify antigen and are activated, changes take place in the cell surface expression of certain receptors and molecules involved in the homing process (Hart et al., 2010).



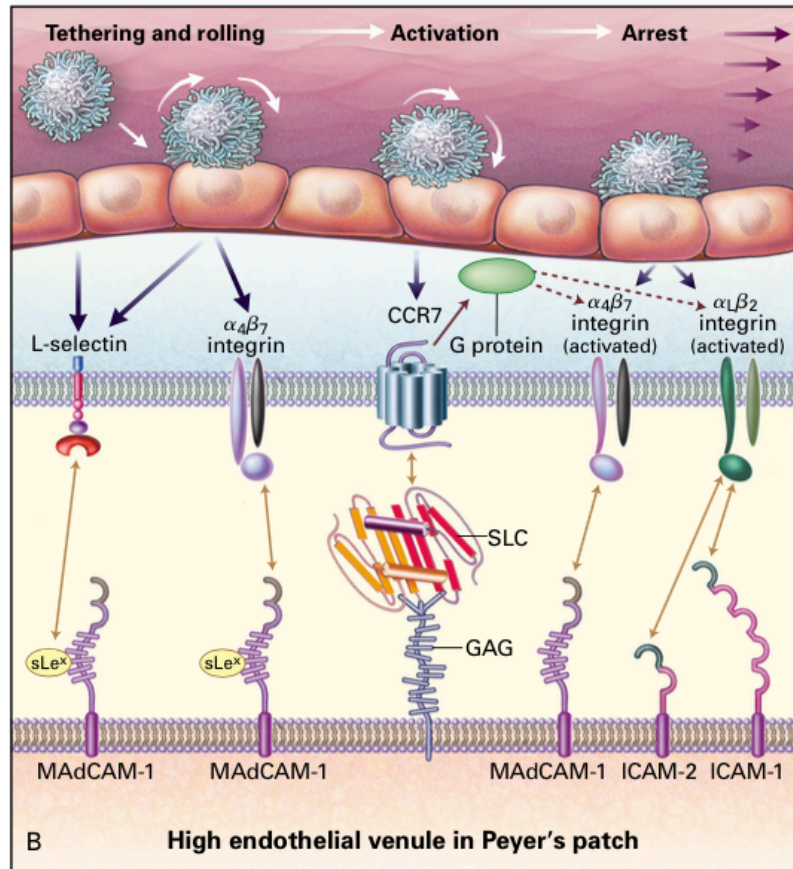


Figure 2. Schematic of the multistep adhesion cascade. Adhesion molecules and receptors involved in lymphocyte homing. (a) Homing in lymph nodes. (b) Homing in Peyer's Patch. Image from von Andrian & Mackay, (2000).

The first step of the multistep adhesion cascade encompasses receptors located on the surface of lymphocytes and on ECs called selectins that bind to interact with counter receptors on the cellular partner. Selectin molecules engage in low affinity interactions with HEV glycoproteins called addressins, ultimately allowing the lymphocyte to roll along the endothelium (Figure 2) (von Andrian & Mackay, 2000). Lymphocyte subsets have the capacity to distinguish between HEVs in peripheral versus mucosal lymph nodes based, in part, on the expression of specific homing receptors. In addition, HEV tissue-

specific expression of vascular addressins also contributes to the capacity of lymphocytes to distinguish different lymphoid organs (Berg, Robinson, Warnock, & Butcher, 1991). HEVs in the peripheral lymph node express the peripheral lymph node addressin (PNAd), and lack mucosal addressin cell adhesion molecule 1 (MAdCAM-1) that is expressed on HEVs in mesenteric lymph nodes and Peyer's Patches (von Andrian & M'Rini, 1998). These differences in addressin molecules create a recognizable distinction between the peripheral lymph nodes, mesenteric lymph nodes, and Peyer's Patches. Thus, this divided expression of specific addressin molecules on HEVs is partially responsible for promoting the directed migration of lymphocytes to distinct lymph nodes (Butcher, Williams, Youngman, Rott, & Briskin, 1999).

Lymphocyte rolling along the endothelium is initiated and mediated by selectin binding with carbohydrate-based ligands (Figure 2). This interaction allows the lymphocyte to be in closer proximity to activating chemokines found on the surface of HEVs (von Andrian & Mackay, 2000). Subsequent cell signaling induces the activation of a conformational change in integrin proteins into their active state. Integrins specifically function to support firm adhesion of the lymphocyte to the endothelial wall. In addition, the selective expression of integrin molecules on the surface of lymphocytes can play a role in regulating lymphocyte trafficking.

Stronger adhesive interactions between the HEV and the patrolling immune cell provided by integrin activation allow the cell to go from rolling along the endothelium to ultimately sticking. Similar cascades are seen when immune cells are homing to non-lymphoid organs. In such cases the venular endothelium is responsible for engaging in

immune cell trafficking by upregulating the appropriate adhesion molecules necessary for the multistep adhesion cascade (Hart et al., 2010).

General Review of Glycoprotein 130

For years the cytokine receptor signaling subunit gp130 has been a target of scientific study due to its association with many different pathologies, such as its expression in tumor cell environments (Xu & Neamati, 2013). Functional analysis of gp130 reveals that it is a signaling subunit on the receptors for the IL-6 family of cytokines (Kishimoto, Akira, Narazaki, & Taga, 1995a; Taga, 1996), (Figure 3). The IL-6 family of cytokines includes IL-6, IL-11, IL-27, oncostatin M (OSM), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), and recently introduced, neuropoietin (NPN) (Figure 3) (Taga & Kishimoto, 1997), (Vlotides, Zitzmann, Stalla, & Auernhammer, 2004), (Derouet et al., 2004). Since these signaling proteins all share the common signal transducer it is not surprising that they have potential overlaps in function (Fasnacht & Müller, 2008). Partial heterogeneity in the signaling can be seen by the different protein cytokines based on some requiring gp130 subunits to be heterodimers or homodimers for proper signal transduction (Fasnacht & Müller, 2008; Kishimoto, Tanaka, Yoshida, Akira, & Taga, 1995b).

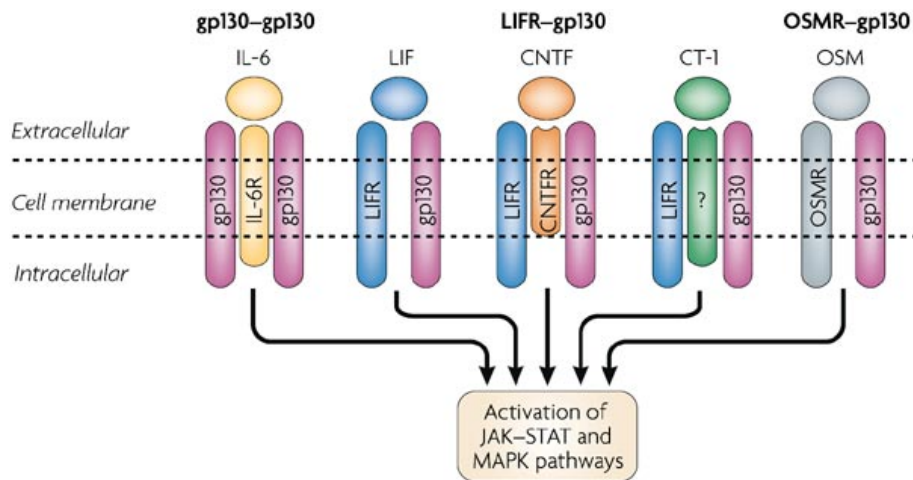


Figure 3. Schematic of the cytokine receptor subunit glycoprotein 130 signaling. Display of the different ligands known to interact with the signaling subunit gp130 in a homodimer or heterodimer conformation with other various receptor subunits. The interleukin-6 (IL-6) ligands associate with gp130 homodimers. The gp130 heterodimer associated receptor signaling includes the ligands leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophonin 1 (CT-1) and oncostatin M (OSM). These cytokines recognized by different combinations of the various receptor subunits (IL-6R, LIFR, CNTFR, OSMR) form complexes with the gp130 subunit and mediate signaling pathways that are involved with intercellular activation of the Janus-activated kinase-signal transducer and activator of transcription (JAK-STAT) and the mitogen-activated protein kinase (MAPK) pathways. Image is from (Bauer, Kerr, & Patterson, 2007).

Straight knockout mice that lack the gp130 gene in all cells show early lethality at birth or shortly after, about 12.5 days depending on the background of mice (Yoshida et al., 1996). This highlights gp130's salient role in development. Experiments have also been performed that have postnatally induced deletion or inactivation of the gp130 gene in different mouse models. Such postnatal deletion results in many defects including neurological, cardiac, hematopoietic, immunological, hepatic and pulmonary (Betz et al., 1998).

Conditional KO mouse models

In the mouse models we use, a gene targeted for deletion is floxed when it is flanked by two *loxP* sequences (St-Onge, Furth, & Gruss, 1996). This targets the gene to be consequently excised by the Cre-recombinase enzyme, creating the conditional knockout mouse (Figure 4). In order to have an organ-specific or cell-specific deletion, the Cre-recombinase enzyme is under the control of a cell specific promoter of choice (Kohan, 2008).

The McEver lab used such a conditional mouse model to study the role of gp130 in bone marrow. They produced a conditional knockout strain that lacked gp130 selectively on hematopoietic and endothelial cells (Yao et. al., 2005). This was accomplished through the use of the Tie2 promoter/enhancer to drive Cre expression, knowing that Tie2 is an endothelial cell marker and transiently expressed in hematopoietic stem cells (Yao, Yokota, Xia, Kincade, & McEver, 2005). Tie2Cre⁺/gp130^{fl/fl} mice specifically lack gp130 on the endothelial and hematopoietic compartments and thus are a suitable model to study the role of gp130 in homing.

VE-Cadherin (VE-Cad) is an intercellular junction protein only expressed on endothelial cells. Thus the VE-Cad promoter can be used to induce expression of tetracycline Transactivator protein (tTA) selectively in endothelium (St-Onge et al., 1996). tTA, under control of the VE-Cad promoter in the inducible conditional knockout mouse model will activate the tet-operator (tet-O). In turn, the element tet-O promoter drives expression of the Cre-recombinase gene resulting in endothelium specific deletion of floxed genes (St-Onge et al., 1996).

There are two known inducible conditional knock out systems, the ‘tet-off’ and ‘tet-on’ system, both regulated by an exogenous ‘trigger’ molecule, doxycycline (DOX) (Figure 4) (Baron & Bujard, 2000). For our purposes, when gene deletion is to occur, administration of DOX is stopped, allowing for the tTA to bind the VE-Cad- tet-O promoter to drive endothelial Cre expression resulting in deletion of the floxed gp130 gene (Figure 4). It is important to note that the establishment of the VE-Cad-gp130 KO mouse model provides control on the timing of gene deletion and allows for endothelial-specific deletion to occur in contrast to the Tie2Cre-gp130 KO that exhibits loss of gp130 on the endothelial and hematopoietic compartment starting in the prenatal phase.

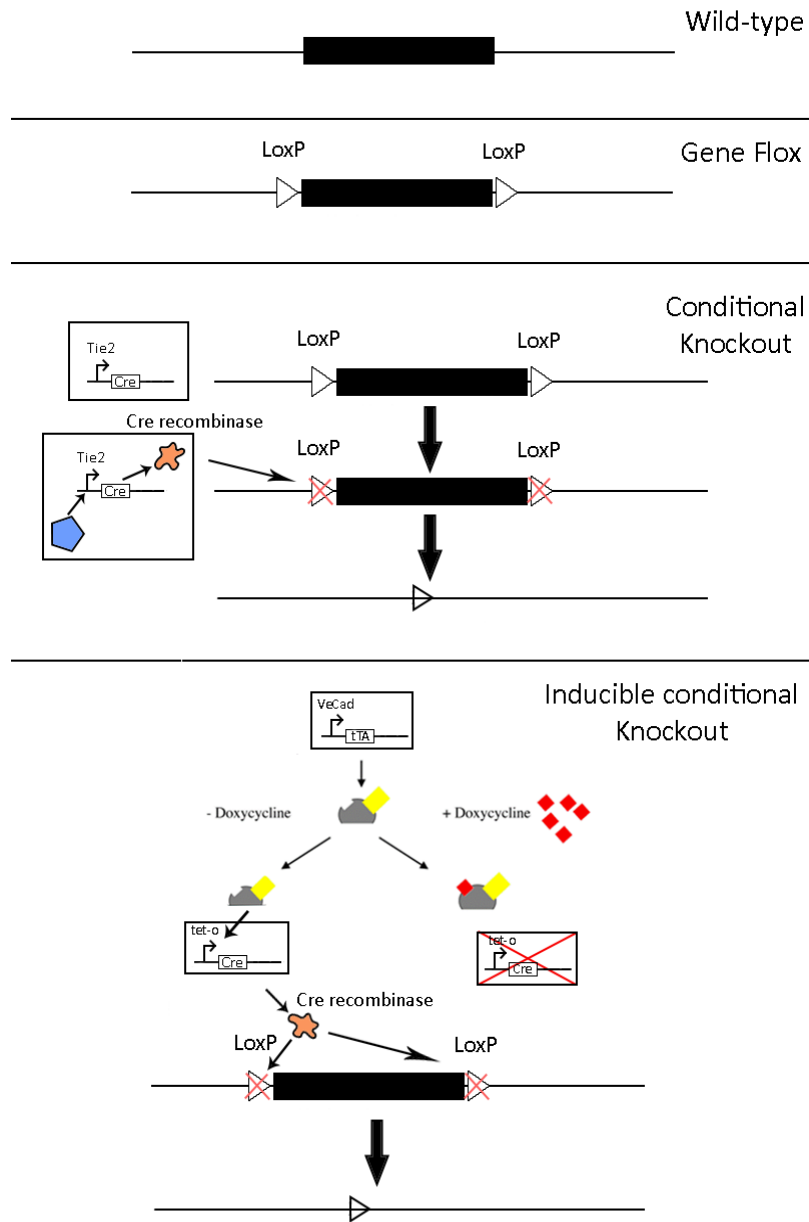


Figure 4. Schematic representation of conditional knockout mouse models. (a) The conditional knockout model with the target gene (black rectangle) flanked by two *loxP* sequences. Floxed genes are subsequently excised by the Cre-recombinase, resulting in gene deletion as depicted. (b) The inducible conditional knockout model with and without doxycycline (DOX) systems. Image depicts tet-off system that showing that upon DOX removal Cre-recombinase is expressed and results in gene deletion, specifically in the endothelial cells. Image courtesy of Blanche Gauthier.

The Present Study

Ensuing prior transcriptome analysis of venular endothelial, non-venular endothelial and lymphatic endothelial cell subsets done by Dr. A. Thiriot in the von Andrian lab, it was discovered that gp130 is overexpressed on venules relative to non-venules in all tissues studied including lymph node (LN), skin, adipose tissue and colon. As venular endothelial cells are known for engaging in lymphocyte trafficking via the multistep adhesion cascade, collection of this data evoked the question of gp130's role in homing.

Although many transgenic knockout models studying the effects of gp130 expression have shown the importance of the signaling subunit in many different tissue and cell types, the role of gp130 in lymphocyte homing, at steady state or under inflammatory conditions in adult tissue, remains to be identified.

In order to efficiently study the role of gp130 in lymphocyte homing, selective deletion of gp130 needs to occur in cell types associated with lymphocyte trafficking. Thus, we used a conditional knockout (Tie2Cre-gp130 KO) and an inducible conditional knock (VE-Cad-gp130 KO) out system to provide us with mouse models that have cell-specific deletions of gp130.

We explored the role of gp130 in the homing of immune cells using both mouse models. To address this, the described mouse models with modified expression of gp130 were utilized and subsequent homing assays were performed. These data provide evidence that gp130-dependent signaling in endothelial and hematopoietic cells contributes significantly to lymphocyte homing.

Our first goal was to identify any role that gp130 expression may have in lymphocyte trafficking. Next, given confirmation of the necessity of gp130 expression for functional lymphocyte recruitment to some SLOs, we wanted to understand the effects of gp130 expression on lymphocyte trafficking. Specifically, we aimed to identify the cell types responsible for engaging with the signaling subunit and to recognize those cell types required to express the signaling subunit during lymphocyte homing. Taken together, in this project we investigated the potential role of gp130 in lymphocyte homing in order to better understand lymphocyte recruitment dynamics at steady state or during inflammatory settings.

Specific Aims

Taking advantage of these the conditional knockout and inducible conditional knockout mouse models, we have presumed the following project aims:

- 1) In order to investigate the role of glycoprotein 130 in lymphocyte homing we have:
 - a. Evaluated novel phenotypes of mice deficient in gp130 on endothelial and hematopoietic cells using a conditional knockout mouse model (Tie2Cre-gp130 KO) and characterize the composition of the lymphoid organs.
 - b. Evaluated any defects in homing of donor β -actin-GFP splenocytes in Tie2Cre-gp130 KO, Tie2Cre-gp130 Het and Tie2Cre-gp130 WT recipients by performing a short-term homing assay.

- 2) To determine the molecular mechanism behind endothelial gp130's involvement in lymphocyte homing to secondary lymphoid organs we have:
 - a. Established and characterize an inducible conditional knockout mouse model (VE-Cad-gp130 KO).
 - b. Examine the cellularity and composition of different organs to detect any modifications.
 - c. Evaluated lymphocyte homing capabilities in these mice by performing a short-term homing experiment.

METHODS

Animal Model

All experiments were performed in accordance with the National Institute of Health guidelines and all mouse protocols were approved by the Institutional Animal Care and Use Committee of Harvard Medical School. The C57BL/6 mice used in this paper came from JAX Labs and the Charles River Laboratory. Experimental knockout strains were either made or maintained as breeders by careful breeding strategies and specific crossings of different strains of mice, all with a C57BL/6 background.

Conditional gp130 knockout mice that are lacking gp130 in the endothelial cell and hematopoietic compartment were Tie2Cre⁺/gp130^{fl/fl} and gp130^{fl/fl} mice were provided by Dr. Roger P. McEver (Oklahoma Medical Research Foundation). Genotypes of mice were assessed via polymerase chain reaction (PCR) on genomic DNA. Conditional knockouts are distinguished by their Tie2Cre⁺/gp130^{fl/fl} genotype. Control mice for our experiments were littermates of the knockouts and recognized by genotype analysis as being Tie2Cre⁻/gp130^{fl/fl} or Tie2Cre⁺/gp130^{+/+} since they shared the same phenotype of wild type mice by expressing gp130 without any modifications.

The inducible conditional knockout mouse model under control of the VE-Cad promoter was created in our laboratory's animal facility. VE-Cad⁺/tTA⁺ and tet-OCre⁺ mice were provided by Dr. Laura Benjamin and Tanya Mayadas. VE-Cad⁺/tTA⁺/tet-OCre⁺ mice were bred with gp130^{fl/fl} to create the inducible conditional knockout. Doxycycline (Bio-serv) (DOX) was administered via diet at the dose concentration of 200mg/kg. DOX was removed and replaced with a normal non-fat p53 diet from the

inducible conditional knockout animals at the time of birth. The wild-type, heterozygous floxed, and homozygous floxed gp130 alleles were detected by genotyping on genomic DNA. Primers for the genes are listed.

Gp130 gene: gp130 A: 5' ACG TCA CAG AGC TGA GTG ATG CAC-3' , gp130 B: 5' GGC TTT TCC TCT GGT TCT TG-3' , gp130 floxed allele =550bp, WT allele = 400bp.

Cre gene : Cre A: 5' AGG TGT AGA GAA GGC ACT TAG C-3' , Cre B: 5' CTA ATC GCC ATC TTC CAG CAG G-3' , Cre allele =400bp, WT allele = not detected.

tTA gene: tTA Fwd 5'- GAC GCC TTA GCC ATT GAG AT -3' , tTA Rvs 5'- CAG TAG TAG GTG TTT CCC TTT CTT -3' , tTA allele = 350 bp, WT allele = not detected

In these knockout systems, heterozygous expression of the Cre-recombinase enzyme is sufficient for excision of a floxed gene, therefore homozygous or heterozygous expression of the Cre-recombinase gene is not distinguished in this paper.

Antibodies

For immunohistology and flow cytometry analysis, the following antibodies were used: anti-glycoprotein 130/gp130/CD130 (eBioscience, clone K6P130), anti-CD31 (clone 390), anti-CD45.1 (clone A20), anti-CD45.2 (clone 104), anti-TCR β (H57-597), anti-CD19 (clone 6D5), anti-NKp46/CD335 (clone 29A1.4), anti-CD3 (clone 17A2), anti-gp38/podoplanin (clone 8.1.1), 7-AAD cell viability dye from Biolegend.

Short-term Homing Assay

Cell suspension of β -actin-GFP cells was prepared by harvesting the spleen of a β -actin-GFP reporter mouse and subsequently isolating splenocytes. Spleen was homogenized and treated with red blood cell lysis buffer for one minute. The reaction was then quenched with five milliliters of HBSS media with 2% fetal bovine serum (FBS). Trypan blue-negative live cells were counted using a hemocytometer. Cell suspension of 10×10^6 cells/200ul was prepared and injected intravenously by tail vein per mouse. This cell suspension input was then counted using the Acurri (BD) and analyzed with Flow cytometry (FACS Canto (BD)) for GFP+ expression. Mice were sequentially sacrificed 1.5 hours after injection. Mouse pool of peripheral lymph nodes (inguinal, axillary and brachial), spleen, mesenteric lymph nodes, and Peyer's patch organs were harvested, homogenized, prepared via filtering, stained and analyzed by FACS for the presence of GFP+ cells.

Half the peripheral lymph nodes were subjected to an endothelial cell isolation harvest, and stained and analyzed via FACS for expression of gp130 for the VE-Cad-gp130 strain.

Statistical analysis:

All data are presented as mean \pm SEM unless noted otherwise. Statistical analysis used Student *t*-Test and one-way ANOVA accordingly.

RESULTS

To determine the role of glycoprotein 130 (gp130) in lymphocyte homing, we used a mouse model that has specific deletion of gp130 in endothelial cells (ECs). Here the conditional knockout mouse Tie2Cre-gp130 was evaluated for novel phenotypes and lymphocyte homing deficiencies.

Total number of hematopoietic (CD45+) cells, were counted in the lymphoid organs of Tie2Cre-gp130 WT, Tie2Cre-gp130 Het, and Tie2Cre-gp130 KO mice. In the blood, cellularity was increased significantly in Tie2Cre-gp130 KO mice compared to Tie2Cre-gp130 WT or Tie2Cre-gp130 Het, with no differences distinguished when comparing the Tie2Cre-gp130 WT with Tie2Cre-gp130 Het blood CD45+ cell counts (Figure 5). This trend is amplified in the spleen, where total hematopoietic cell numbers in Tie2Cre-gp130 KO mice is significantly higher compared to Tie2Cre-gp130 WT or Tie2Cre-gp130 Het mice (Figure 5). This enhanced cellularity demonstrates noticeable splenomegaly, an observation in accordance with published data by (Yao, Yokota, Xia, Kincade, & McEver, 2005), characterizing the phenotypes of these mice.

The total number of CD45+ cells in peripheral lymph nodes (PLNs) are significantly reduced but not abolished in Tie2Cre-gp130 KO mice compared to Tie2Cre-gp130 WT (Figure 5), and there were no significant differences between the WT and Tie2Cre-gp130 Het mice (Figure 5). Hematopoietic cell counts in cervical lymph nodes (cLN) and auricular lymph nodes (aurLN) also demonstrated significant decreased numbers of hematopoietic cells in the Tie2Cre-gp130 KO compared to Tie2Cre-gp130 Het used as control (Figure 5).

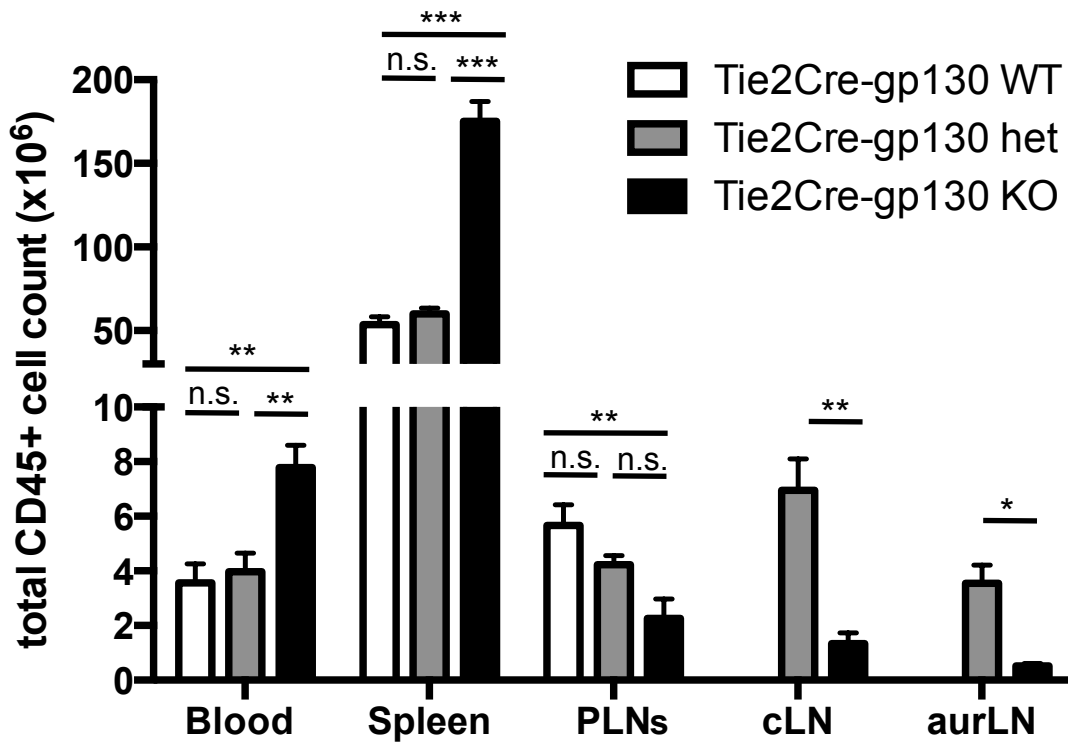


Figure 5. Abolished cellularity in Tie2Cre-gp130 KO blood and secondary lymphoid organs. Total number of hematopoietic cells (CD45+) from various secondary lymphoid organs in Tie2Cre-gp130 WT (white), Tie2Cre-gp130 Het (grey) and Tie2Cre-gp130 KO (black) mice. Total CD45+ cell counts were taken from the blood and the following secondary lymphoid organs: spleen, peripheral lymph nodes (PLNs, pool of inguinal lymph nodes, brachial lymph nodes, and axillary lymph nodes), cervical lymph nodes (cLN), auricular lymph nodes (aurLN), in Tie2Cre-gp130 WT, Tie2Cre-gp130 Het, and Tie2Cre-gp130 KO mice. Blood is expressed as number of CD45+cells/ml of blood. In blood, spleen and PLNs, n=8 mice in Tie2Cre-gp130 WT genotype, n=6 mice in the Tie2Cre-gp130 het genotype, n=10 mice in the Tie2Cre-gp130 KO genotype. One-way ANOVA, n.s., not significant. *, P<0.05, **, P<0.01, ***, P<0.0001. In cLN and aurLN n=3 mice in the Tie2Cre-gp130 Het genotype, n=3 mice in the Tie2Cre-gp130 KO genotype. Student *t*-Test, n.s., not significant. *, P<0.05, **, P<0.01. Data are shown as mean \pm SEM.

Knowing that gp130 is expressed on endothelial and hematopoietic cells of wild-type but not the conditional knockout strains based on experimental data from (Yao et al., 2005), we anticipated that the deficiency of gp130 would affect lymphocyte migration into lymphoid organs. To address this, a short-term homing assay was performed. For this purpose, splenocytes from a transgenic β -actin-GFP reporter mouse were isolated and injected intravenously into recipient Tie2Cre-gp130 WT, Tie2Cre-gp130 Het, and Tie2Cre-gp130 KO mice. 1.5 hours after injection, we quantified the number of GFP+ cells in secondary lymphoid organs such as spleen, PLNs, and MLNs, counted by FACS. Donor GFP+ cells that had accumulated in recipient lymphoid organs were counted using flow cytometry (Uchimura et al., 2005). We used the spleens as organ controls given that lymphocyte recruitment to the spleen does not follow the multistep adhesion cascade mechanism.

Blood samples and homing of donor cells to recipient spleens were comparable in all three strains, as expected (Figure 6). In the Tie2Cre-gp130 KO mice, we detected a significant reduction of GFP+ homed cells in both PLNs and MLNs as compared to both Tie2Cre-gp130 WT and Tie2Cre-gp130 Het mice (Figure 6). Such effects of diminished homing were not seen when comparing the Tie2Cre-gp130 Het to Tie2Cre-gp130 WT mice (Figure 6).

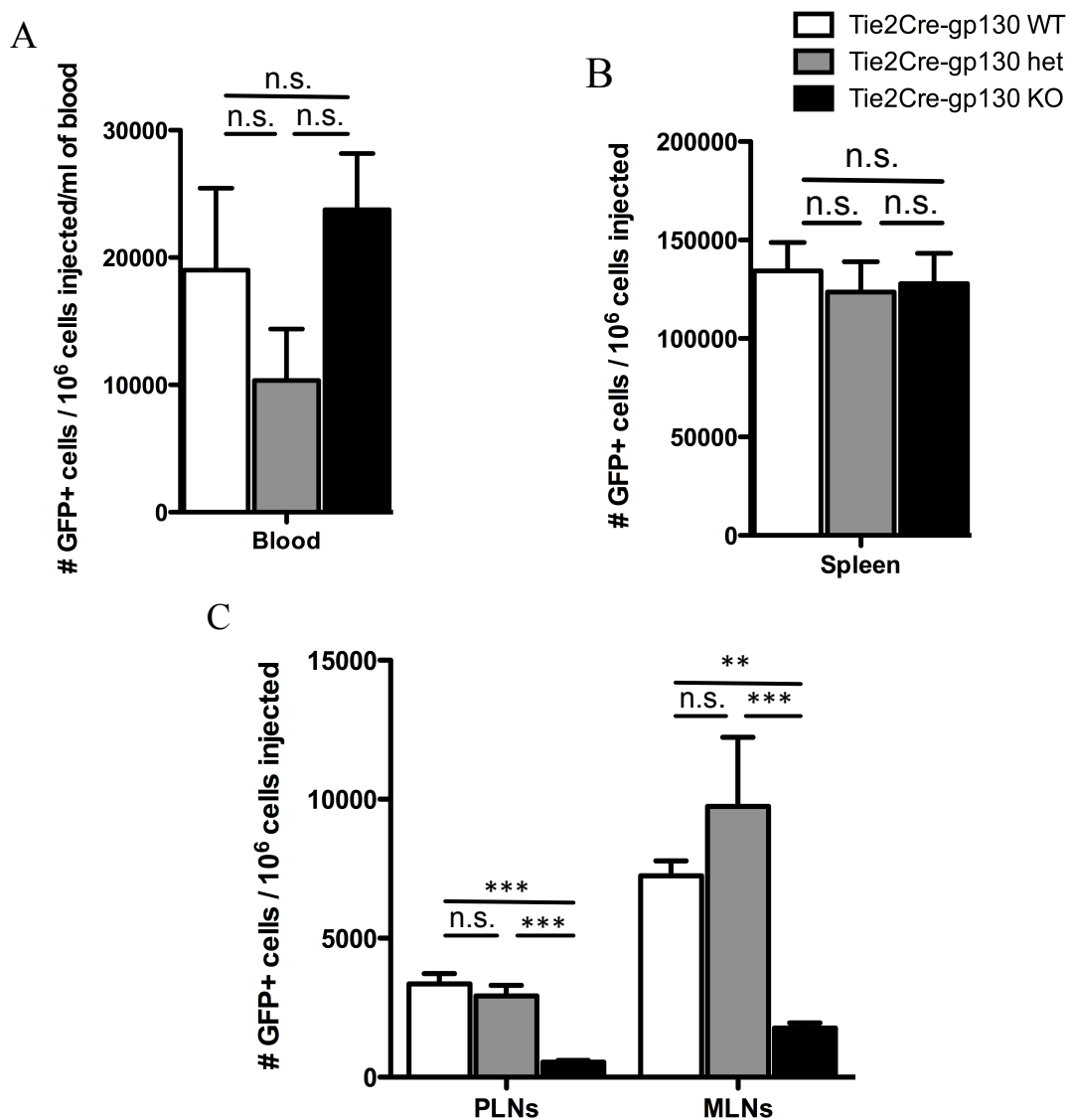


Figure 6. Reduced homing capabilities of β -actin-GFP splenocytes in Tie2Cre-gp130 KO mice. Splenocytes from β -actin-GFP mice were injected intravenously into age matched Tie2Cre-gp130 WT (white), Tie2Cre-gp130 Het (grey) and Tie2Cre-gp130 KO (black) mice. Splenocytes derived from β -actin-GFP transgenic mice were detected in (a) blood, (b) spleen and (c) peripheral lymph nodes (PLNs, pool of inguinal lymph nodes, brachial lymph nodes, and axillary lymph nodes), and mesenteric lymph nodes (MLNs) of each mouse as analyzed by flow cytometry. The distribution of GFP+ cells was analyzed 1.5 hours after tail vein injection and is expressed as number of GFP+ cells/ 10^6 cells injected, gated among lymphocytes in each organ. Blood is expressed as the number of GFP+ cells/ 10^6 cells injected/ml of blood. n=8 mice in the Tie2Cre-gp130 WT genotype, n=6 mice in the Tie2Cre-gp130 Het genotype, n=10 mice in the Tie2Cre-gp130 KO genotype. One-way ANOVA n.s., not significant, **, P<0.01. ***, P<0.0001. Data are shown as mean \pm SEM.

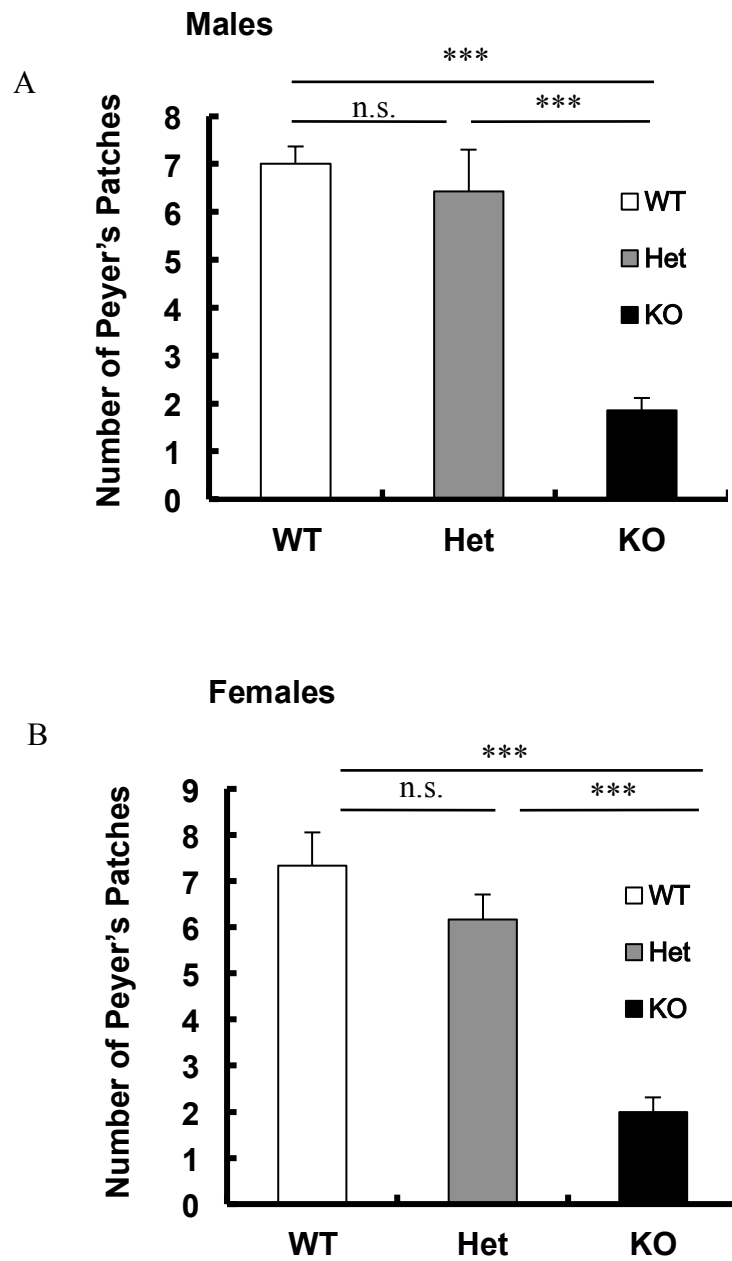


Figure 7. Tie2Cre-gp130 KO mice exhibit a reduced number of Peyer's Patches regardless of sex. Number of Peyer's Patches found in (a) male and (b) female mice from Tie2Cre-gp130 WT (white), Tie2Cre-gp130 Het (grey) and Tie2Cre-gp130 KO (black) strains. n=6 to 7 mice of each genotype. One-Way ANOVA n.s., no significance, ***, $P < 0.0001$. Data are shown as mean \pm SEM. Image courtesy of Dr. Yasuhiro Nemoto.

Lymphocyte recruitment to Peyer's Patches could not be assessed because of the unexpected dramatic decrease in PPs number in Tie2Cre-gp130 KO mice (Figure 7). Tie2Cre-gp130 WT and Tie2Cre-gp130 Het mice displayed no differences in the number of Peyer's Patches harvested (Figure 7). Males and females in each group were compared to determine if sex had a role in the exhibited loss of PPs found in the Tie2Cre-gp130 KO, but the results demonstrate that sex of the mouse is not a significant factor in this morphological defect (Figure 7).

To investigate this conspicuous phenotype further and to rule out that the defect in Peyer's Patches could have been an effect resulted from a smaller morphology of the small intestine (SI), measurements of the lengths and weights of the harvested small intestine were recorded (Figure 8A, 8C, 8E). The lengths and weight of the colon were measured for control (Figure 8B, 8D, 8E). No significant differences were seen in the morphologies as measured by the lengths and weights of harvested small intestine in the different mouse strains, confirming that the decrease in the number of PPs is due to developmental defects in this conditional knockout strain (Figure 8).

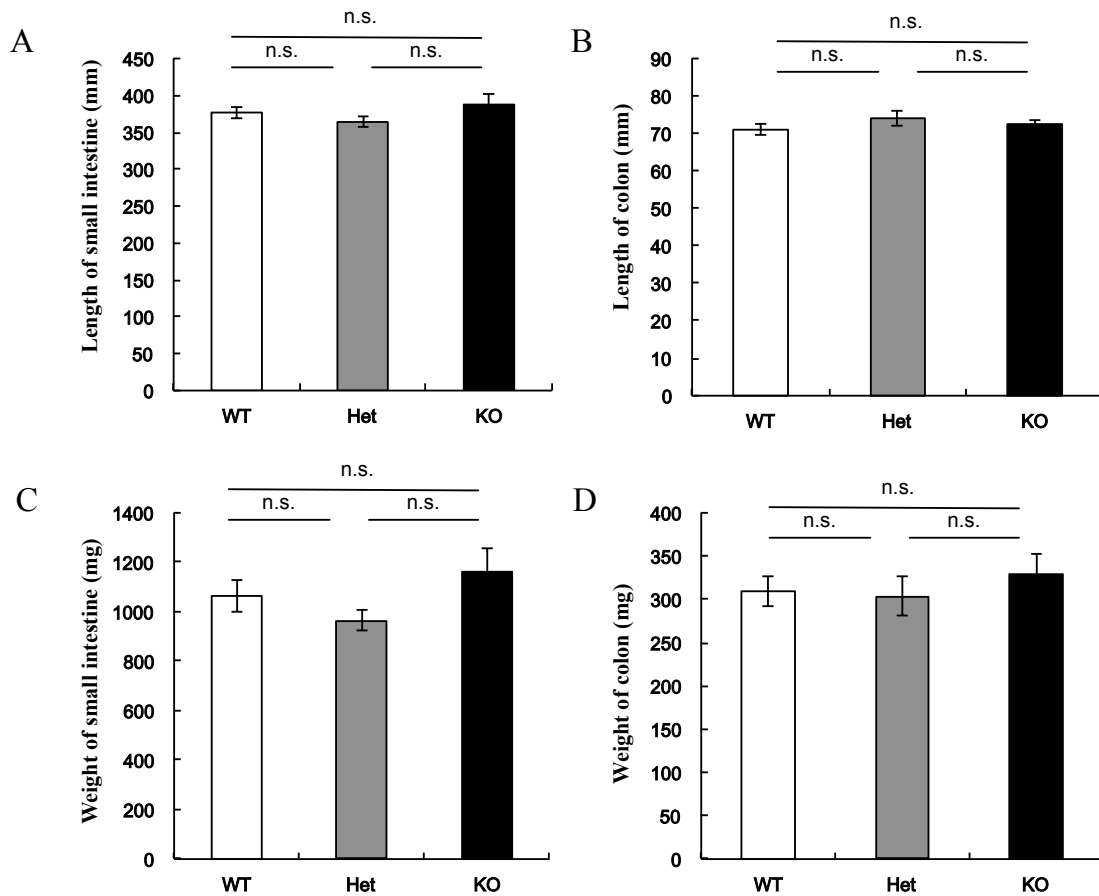


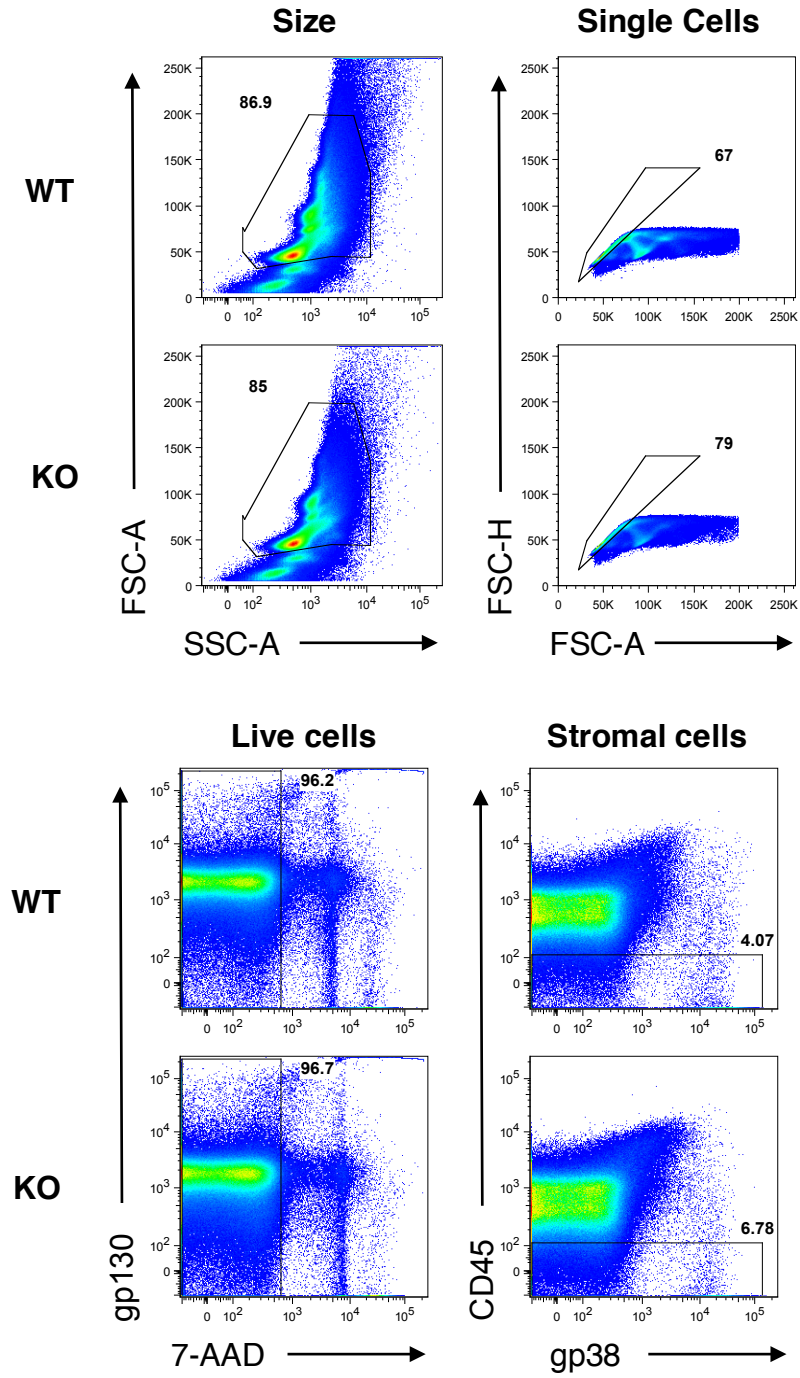
Figure 8. No differences in length or weight of intestines in Tie2Cre-gp130 KO. (a) Length of small intestine measured in mm, (b) length of colon measured in mm, (c) weight of small intestine measured in mg, and (d) weight of colon measured in mg. Measurements were taken in Tie2Cre-gp130 WT, Tie2Cre-gp130 Het and Tie2Cre-gp130 KO mice. n=5 to 8 mice in each genotype. One-Way ANOVA n.s., no significance. Data are shown as mean \pm SEM.

Given the defect in homing capabilities and morphological defects in PPs development, we wanted to understand the biology behind this phenotype in the Tie2Cre-gp130 KO model. Because in Tie2Cre-gp130 KO mice gp130 is lacking in both the endothelial as well as the hematopoietic compartment, we aimed to determine which cell type was responsible for the significant characteristics and phenotypes observed. To address this question, we created an inducible conditional knockout mouse that allowed for the deletion of the gp130 gene to be restricted to the endothelial cell compartment using the VE-Cadherin (VE-Cad) promoter (Figure 4, 9).

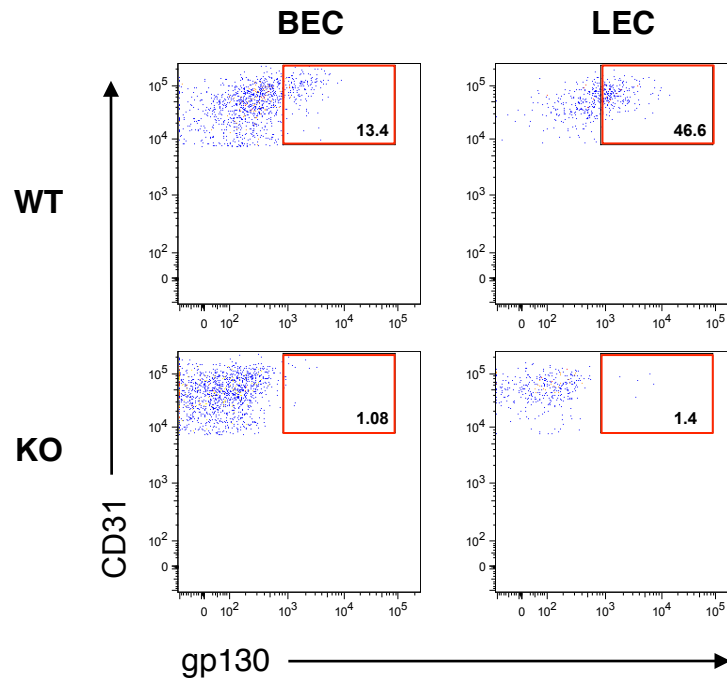
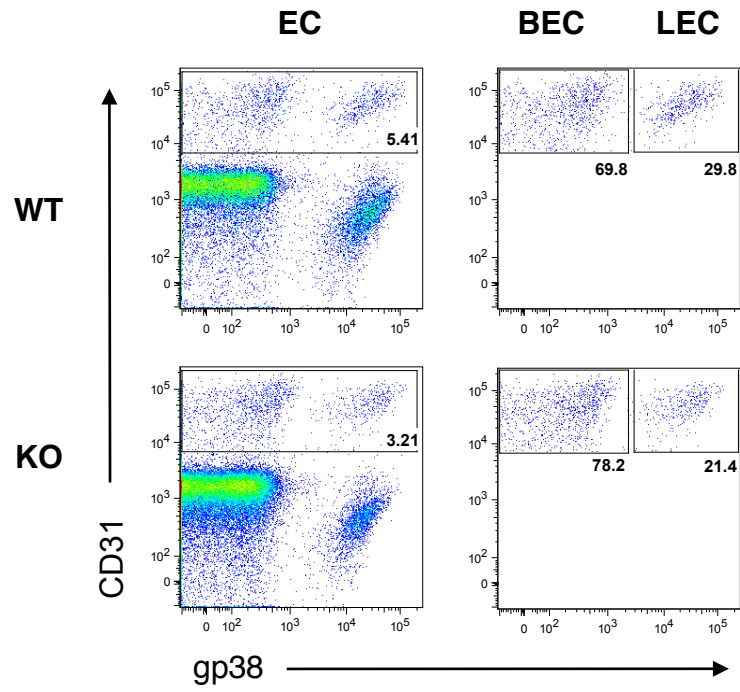
By selectively cross breeding a VE-Cad-tTA⁺/tet-OCre⁺ mouse, with a gp130 floxed (gp130^{fl/fl}) mouse strain we were able to obtain an inducible conditional knockout (VECad-tTA⁺/tet-OCre⁺/gp130^{fl/fl}) (Figure 4). Doxycycline (DOX) was administered via a food diet and was removed at birth. Subsequent experiments were performed at least eight weeks after DOX removal to ensure gp130 gene deletion.

To confirm inducible gene deletion on the endothelium, flow cytometry analysis was performed. Blood and peripheral lymph node samples collected from the VE-Cad-gp130 KO mice showed a lack of gp130 expression on endothelial cells (Figure 9). This data shows that gp130 is absent in both blood endothelial cells (BECs) and lymphatic endothelial cells (LECs) confirming the inducible conditional system for gp130 gene deletion is functional (Figure 4, 9).

A



B



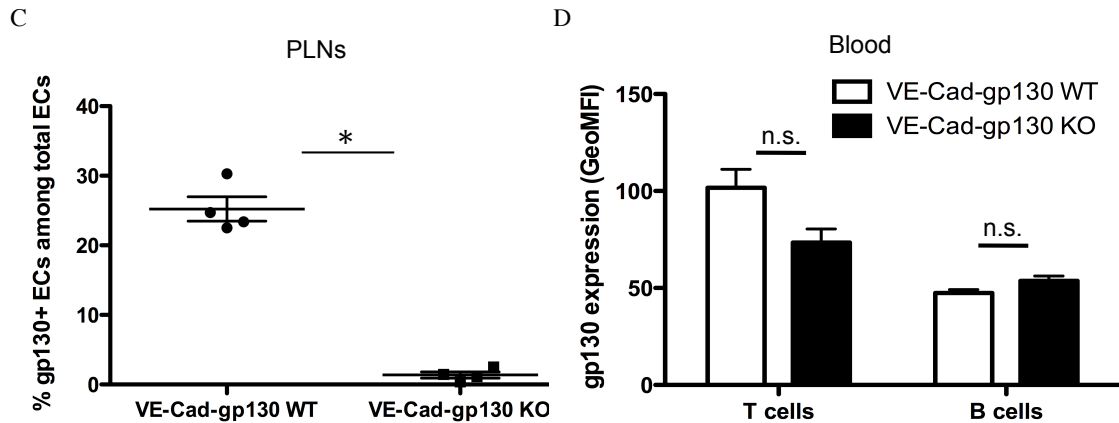


Figure 9. VE-Cad-gp130 KO mice lack gp130 expression only on the endothelial cell compartment. Gating strategies of peripheral lymph node samples analyzed by flow cytometry for expression of gp130 on the endothelium of VE-Cad-gp130 WT (white) and VE-Cad-gp130 KO (black) mice. Our FACS gating strategy is the following: (a) we gate on large population (size) of single cells and live cells (7AAD⁻) cells, we exclude CD45⁺ hematopoietic cells and (b) gate on blood endothelial cells (BEC) as gp38⁺CD31⁺ and lymphatic endothelial cells (LEC) as gp38⁺CD31⁺. BEC and LEC are further differentiated into gp38⁺CD31⁺gp130⁺ and gp38⁺CD31⁺gp130⁻, respectively. (c) Frequency of gp130⁺ endothelial cells among total endothelial cells quantified in peripheral lymph node (PLNs) from Tie2Cre-gp130 WT and Tie2Cre-gp130 KO mice. (d) Geometric mean fluorescent intensity (GeoMFI) expressed in blood samples analyzed by flow cytometry for expression of gp130 on T and B lymphocytes in VE-Cad-gp130 WT and VE-Cad-gp130 KO mice. n=4 mice in each genotype. Student *t*-Test, n.s., no significance, *, P<0.05. Data are shown as mean ± SEM.

VE-Cad-gp130 KO mice did display slight splenomegaly as assessed by the weight of VE-Cad-gp130 KO spleens compared to wild type (Figure 10). In addition, surprisingly, no defect in PPs number was seen in the VE-Cad-gp130 KO mice compared to that of VE-Cad-gp130 WT (Figure 11).

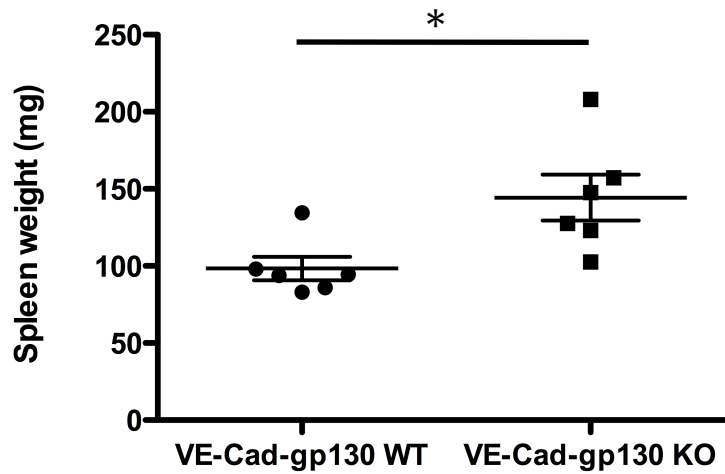


Figure 10. VE-Cad-gp130 KO displays mild splenomegaly. Graph of spleen weights from control VE-Cad-gp130 WT compared to VE-Cad-gp130 KO mice with doxycycline removed for 18-20 weeks. n=8 mice in each genotype. Student *t*-Test, *, P<0.05. Data are shown as mean \pm SEM.

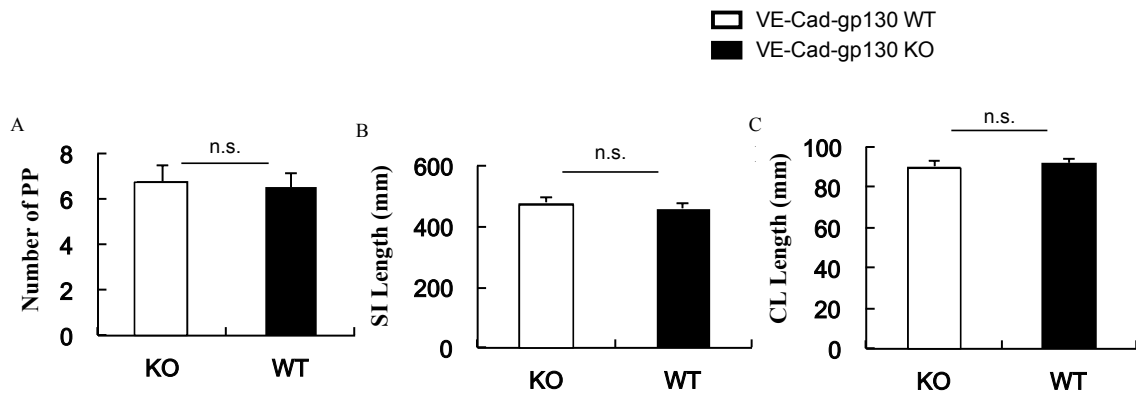


Figure 11. VE-Cad-gp130 KO mice do not lack Peyer's patches. (A) Number of Peyer's Patches (PP) from VE-Cad-gp130 KO and VE-Cad-gp130 WT mice counted. (B) Lengths of small intestine and (C) colon from VE-Cad-gp130 KO and VE-Cad-gp130 WT mice were measured in mm. Student *t*-Test, n.s., no significance. n=4 mice of each genotype. Data are shown as mean \pm SEM.

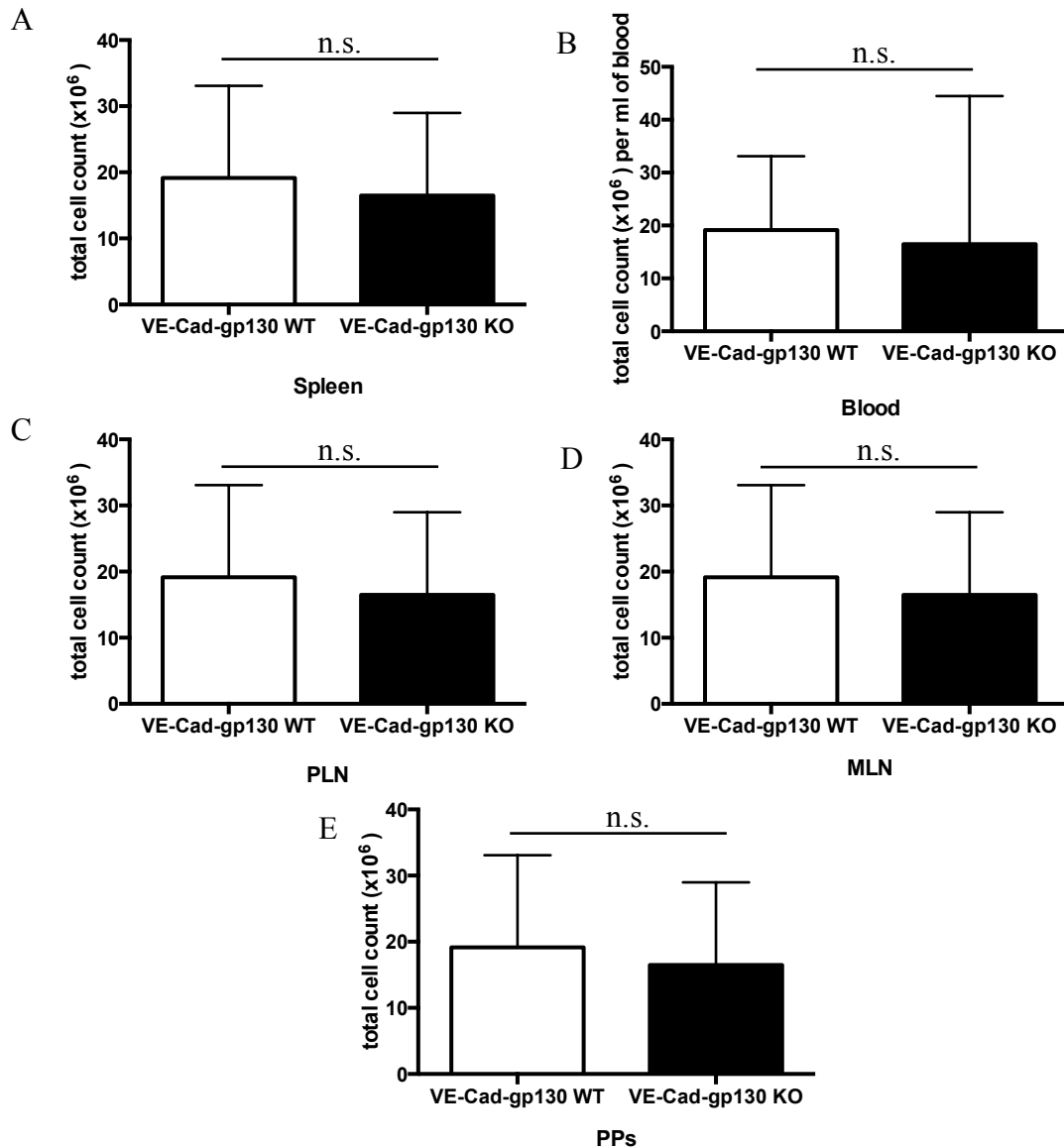


Figure 12. Cellularity in VE-Cad-gp130 KO mice. Total cell counts in various secondary lymphoid organs and blood in VE-Cad-gp130 WT (white) and VE-Cad-gp130 KO (black) mice. Total cell numbers taken from (a) blood and secondary lymphoid organs including (b) spleen, (c) peripheral lymph nodes (PLNs, pool of inguinal lymph nodes, brachial lymph nodes, and axillary lymph nodes), (d) mesenteric lymph nodes (MLNs), and (e) Peyer's Patches (PPs), from VE-Cad-gp130 WT and in VE-Cad-gp130 KO mice that were removed from doxycycline for 18-20 weeks. Blood is expressed as number of cells/ml of blood. n=4 mice of each genotype. Student *t*-Test, n.s., no significance. Data are shown as mean ± SEM.

Having confirmed that gp130 is expressed in endothelium of VE-Cad-gp130 WT but not VE-Cad-gp130 KO mice, we next asked whether the homing defect observed in the conditional Tie2Cre-gp130 KO mouse persists in the inducible conditional model, addressing if the homing deficiency was due to the lack of gp130 on the endothelium or the hematopoietic compartment. To gain insight into the role of gp130 on the endothelial compartment in lymphocyte trafficking, a short-term homing assay was performed. Following the same homing protocol, splenocytes from a transgenic β -actin-GFP reporter mouse were isolated and injected intravenously into recipient VE-Cad-gp130 WT and VE-Cad-gp130 KO mice. 1.5 hours after cell injection, we quantified donor GFP⁺ cells that had accumulated in recipient lymphoid organs using flow cytometry, with blood and spleen samples taken as controls (Uchimura et al., 2005).

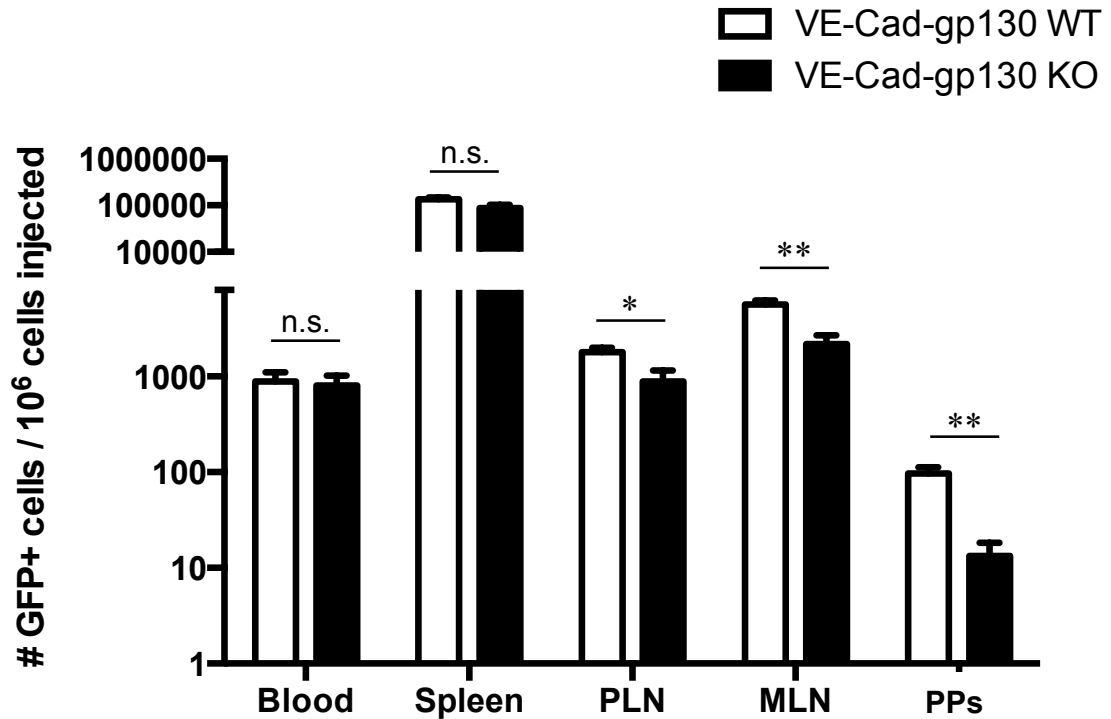


Figure 13. Lymphocyte recruitment is impaired in VE-Cad-gp130 KO mice. Homing of β -actin-GFP splenocytes in VE-Cad-gp130 WT (white) and in VE-Cad-gp130 KO (black) mice. Splenocytes derived from β -actin-GFP⁺ transgenic mice homing to peripheral lymph nodes (PLNs, pool of inguinal lymph nodes, brachial lymph nodes, and axillary lymph nodes), mesenteric lymph nodes (MLNs), Peyer's Patches (PPs) and spleen in VE-Cad-gp130 WT and in VE-Cad-gp130 KO mice that were removed from doxycycline for 18-20 weeks. The distribution of GFP⁺ cells was analyzed 1.5 hours after tail vein injection and expressed as the number of GFP⁺ cells/ 10^6 cells injected, gated among lymphocytes in each lymphoid organ. Blood is expressed as number of GFP⁺ cells/ 10^6 cells injected /ml of blood. PPs is expressed as number of GFP⁺ cells/ 10^6 cells injected / Peyer's patch. n=4 mice in each genotype. One-way ANOVA, n.s., no significance. *, P<0.05, **, P<0.01. Data are shown as mean \pm SEM.

VE-Cad-gp130 KO mice have lymphocyte homing reduced in the peripheral lymph nodes compared to VE-Cad-gp130 WT recipients similar to what was seen in the Tie2Cre-gp130 KO mice (Figure 13). Homing to mesenteric lymph nodes was also significantly diminished in the VE-Cad-gp130 KO mice compared to VE-Cad-gp130 WT recipients (Figure 13). Homing to Peyer's Patches was severely impaired in the VE-Cad-gp130 KO recipient mice (Figure 13). Blood samples and homing of donor splenocytes to recipient spleen organs were comparable in VE-Cad-gp130 KO mice to that of VE-Cad-gp130 WT, as expected (Figure 13). These data indicate that gp130 expression does play a role in lymphocyte homing, on the endothelial compartment.

DISCUSSION

The recruitment of lymphocytes from the blood circulation to the lymph nodes is essential to generating an adaptive immune response and maintaining an immune surveillance program during an inflammatory episode. Here we provide *in vivo* evidence for the critical role of the signaling subunit glycoprotein 130 (gp130) in the homing of lymphocytes under steady state conditions by analyzing conditional and inducible conditional knockout mouse models. We report that the absence of the cytokine receptor signaling subunit glycoprotein 130 on endothelial and hematopoietic cells in the conditional knockout (Tie2Cre-gp130 KO) and on endothelial cells only in the inducible conditional knockout mice (VE-Cad-gp130 KO) results in lymphocyte homing deficiencies. In addition to the impaired homing of lymphocytes, unexpected differences in secondary lymphoid organ compositions observed in the two strains lead us to propose that gp130-signaling plays at least two distinct roles in cellular recruitment dynamics; one to be on the level of the endothelium for lymphocyte homing and the other, we hypothesize, on the level of hematopoietic cells for stem cell precursor recruitment.

First, Tie2Cre-gp130 KO and VE-Cad-gp130 KO mice have significant lymphocyte homing deficiencies across all lymph nodes tested, such as peripheral lymph nodes and mesenteric lymph nodes, suggesting that the loss of gp130 on endothelial cells impairs recruitment of lymphocytes to lymph nodes.

Second, Tie2Cre-gp130 KO mice display reduced numbers of Peyer's Patches without any reduction or absence in other secondary lymphoid organs, suggesting that gp130-mediated signaling, on the endothelial and/or hematopoietic compartment, is

critical for the development of Peyer's Patches. In contrast, VE-Cad-gp130 KO mice display no impairment in Peyer's patch development, despite the lack of gp130 on the endothelium. This suggests that the absence of Peyer's Patches in Tie2Cre-gp130 KO mice is a phenotype related to gp130-signaling at the level of the hematopoietic compartment.

In addition, Tie2Cre-gp130 KO mice display a significant reduction in total hematopoietic cell number within the various lymph nodes tested, while lymphocyte numbers in blood and spleen were enhanced, a phenotype not shared by the VE-Cad-gp130 KO strain. It is unlikely that the reduced hematopoietic cell number observed in Tie2Cre-gp130 KO mice causes the reduction in lymphocyte homing capabilities. This interpretation is also supported by the VE-Cad-gp130 KO mice that did not have significant decreases in their hematopoietic cell numbers yet displayed similar impairments in lymphocyte homing. Thus, we favor the notion that the impaired lymphocyte homing capabilities in Tie2Cre-gp130 KO lymph nodes does not cause the reduced number of hematopoietic cells found in these organs. Furthermore, VE-Cad-gp130 KO mice did not share a decreased cellularity in lymph nodes, as seen in Tie2Cre-gp130 KO mice, suggesting that the reduced total hematopoietic cell number is attributable to gp130 deletion on the hematopoietic compartment and not the endothelium.

Interestingly, despite the uncommon patterns in Peyer's Patch development between the Tie2Cre-gp130 KO and the VE-Cad-gp130 KO strains, there was a shared characteristic of splenomegaly, albeit milder, in the VE-Cad-gp130 KO mice. This

suggests a dual role for gp130 expression, such that gp130 plays a role in maintaining sufficient lymphocyte trafficking on the level of the endothelium, as well as the hematopoietic compartment, with dual ablation of the signaling subunit creating the severe splenic compensatory response demonstrated by the Tie2Cre-gp130 KO mouse splenomegaly.

Although the mechanism by which the deletion of gp130 mediated signaling decreases lymphocyte homing capabilities is currently unknown, we hypothesize that signaling pathways activated by gp130 may serve to balance the net expression of adhesion molecules and/or chemokines involved in the multistep adhesion cascade. In order to address this, future experiments will identify the role of gp130 at the level of the multistep adhesion cascade. To investigate the adhesion molecules involved in lymphocyte homing present in VE-Cad-gp130 KO and Tie2Cre-gp130 KO mice, we will focus on the systematic analysis of the components of each of the three steps of the multistep adhesion cascade.

To determine the molecular mechanism behind endothelial cell expression of gp130 and its involvement in lymphocyte recruitment we will first investigate the level of expression of the addressin molecules PNAd and MAdCAM-1 specific to HEVs in PLNs and PPs, respectively, as well as other specific adhesion molecules including VCAM-1, ICAM-1, and ICAM-2 (Girard et al., 2012). This will be done in the conditional knockout Tie2Cre-gp130 mice and the inducible conditional knockout VE-Cad-gp130 mice. We will also examine the structure of the lymph nodes by staining for B cell zones and T cell zones. Research by Yao et. al., (2013), studying neutrophil recruitment in Tie2Cre-gp130

KO mice shows that gp130 deficient mice express normal levels of ICAM-1 on the surface of endothelial cells, thus we do not predict any difference in the VE-Cad-gp130 KO strain (Yao et al., 2013).

Next, to directly observe the effect of endothelial cell gp130 deletion in VE-Cad-gp130 KO mice on the rolling of lymphocytes in lymph node HEV, we will employ the use of intravital microscopy (IVM). To investigate rolling deficiencies, we will focus on L-selectin expression on the lymphocyte, and HEV sialomucins (N-glycans, 6-sulpho sialyl Lewis X) that induce rolling (Girard et al., 2012). Following this interaction is cell sticking that is triggered by lymphocyte CCR7 binding to HEV expressed of CCL21. This causes cell-signaling activation of (lymphocyte associated antigen 1) LFA-1 integrin into its active conformation (Warnock, Askari, Butcher, & Andrian, 1998). LFA-1 activated integrin interacts with ICAM-1 and ICAM-2, on the surface of HEVs and the adherent cells crawl for a short distance within the HEV lumen until final transmigration (Girard et al., 2012). We can investigate all of these phases by examining the expression patterns of proteins involved with each step using IVM and confocal imaging analysis (Halin, Mora, Sumen, & Andrian, 2005).

To rule out the probable effects of diminished lymphocyte homing in the VE-Cad-gp130 KO mice due to the lack of blood perfusion in the organ, and therefore attributing the role of gp130 on the multistep adhesion cascade, we will analyze intraluminal shear forces by intravital microscopy. Specifically, analysis of VE-Cad-gp130 KO Peyer's Patch organs will identify if lymphocyte homing deficiency is a result of a default in the multistep adhesion cascade or rather associated with the lack of blood perfusion as a

results of a PPs organ defect. To highlight gp130 involvement with the multistep adhesion cascade, we will start by analyzing the rolling and sticking fractions of the lymphocytes along the high endothelial venules of PPs.

Studies have shown that the alteration of one of the fundamental adhesion molecules or chemokines, on the level of the lymphocyte or venular endothelium, will result in drastic modifications in trafficking capabilities, indicating the fundamental role that such molecules possess. Research by Chen et al., (2004), demonstrates that lymphocyte adhesion activation of L-selectin by fever-range thermal stress depends on IL-6 signaling, in particular the expression of gp130 receptor subunit (Chen et al., 2004). This study also determined that the thermal response involved the specific IL-6 family of cytokines IL-11, LIF and OSM (Chen et al., 2004). In accordance with this observation, we speculate a strong candidate for the gp130-signaling events required for proper lymphocyte homing to be OSM, also known to be involved in angiogenesis and development (Yao, Pan, Setiadi, Patel, & McEver, 1996). In addition, studies by Wojta et al., (2012), on the cytokine signaling receptor explain that OSM induced pluripotent effects on human endothelial cells, with its proliferative signals limited to the microvascular cells, and not recognized as responsive on macrovascular human umbilical venular endothelial cells (HUVEC) as seen by the lack of proliferation following OSM stimulation (Demyanets, Huber, & Wojta, 2012).

The association of gp130 with OSM may link the lymphocyte homing system to organogenesis. As described previously, the loss of gp130 has a significant impact on a variety of biological events. To identify a role of gp130 in the expression of adhesion

molecules, in particular L-selectin and OSM gp130 signaling, further analysis on the expression levels of these molecules involved, as well as functionality assessments must be made.

Furthermore, we will assess the role of steady state gp130 expression on Peyer's Patch organ development on the level of the lymphoid tissue inducer cells (LTi), a cell type responsible for the organogenesis of Peyer's Patches (Cherrier & Eberl, 2012; Strober, 2010). LTi is positive for the hematopoietic marker CD45 and thus targeted by the conditional knockout system, which supports the hypothesis that LTi stem progenitor cells are involved in our gp130 deleted mouse models (Mebius et al., 2001). If in fact gp130 is expressed on these cells, as will be determined with future experiments, this will support the conclusion that gp130 expression on the hematopoietic compartment, not the endothelial cell is responsible for causing the phenotype of defected PPs in the Tie2Cre-gp130 model, and not in the VE-Cad-gp130. Further supporting the hypothesis that this progenitor cell is the cause for the secondary lymphoid organ defects observed is that LTi cells are only known for being involved with the Peyer's patch organogenesis, and not other secondary lymphoid organ development, consistent with our phenotype of only diminished PPs in the conditional knockout (Honda et al., 2001).

The defect in Peyer's Patch number is a novel characteristic of these mice that has not been previously published, although, other transgenic mouse models have been shown to have similar defects in PPs development, such as the CXCL13 deficient mice (Honda et. al., 2001). CXCL13 is a cytokine produced by stromal fibroblastic reticular cells and follicular dendritic cells found in the lymph node cortex. It is involved with the

intra-nodal migration of B lymphocytes, directing the cells via a chemoattractant chemical gradient to the B cell follicles located along the periphery near the sub-capsular sinus (Figure 1). B cells express the CXCR5 receptor for CXCL13, and without this ligand binding interaction B cell follicles will not form thus effecting PPs development. To address this, we will assess the number of B cells as well as the level of expression of associated chemokines such as, CXCL13, in the PPs of the Tie2Cre-gp130 KO mice.

Another transgenic mouse strain lacking PPs is the Wiskott-Aldrich syndrome protein (WASP)- deficient strain (Snapper et al., 2005). WASP has been described to play a role in cellular locomotion through regulating surface receptor signaling to the actin cytoskeleton (Snapper et al., 2005). It has also been shown that lymphocytes have a reduced tethering and migration when WASP-deficient (Snapper et. al., 2005). Also, similar to the VE-Cad-gp130 KO homing characteristics, mice with WASP-deficient lymphocytes are demonstrated to show significantly more impairment in lymphocyte homing to PPs than to other secondary lymphoid organs (Snapper et. al., 2005). To address the probable role of gp130 signaling and its effects on lymphocyte trafficking by way of WASP interaction, we will also analyze the expression patterns of WASP protein in various secondary lymphoid organs.

Finally, to further our understanding of the role of gp130 in the spleen we will identify any defects in structure by performing immunohistochemical analysis for B cell and T cell zones. Imaging will be done with confocal microscopy and staining for splenic lymphoid nodules, periarteriolar lymphoid sheaths and red pulp.

Taken together, we can conclude that the lack of gp130 expression on the endothelium is responsible for the lymphocyte homing deficiencies in both knockout mouse models, while the lack of gp130 on the hematopoietic compartment is responsible for driving physiological abnormalities observed, including decreased cellularity in lymphoid organs, increased cellularity in systemic blood and spleen, as well as a profound defect in PP formation. Further analysis of this potentially dichotomous role of gp130 expression on endothelial cells and hematopoietic cells is necessary, and important to determining the underlying mechanisms by which gp130 plays a role in lymphocyte homing and homeostasis. Gaining a mechanistic understanding of the role that gp130 expression plays on the endothelium and specifically its interactions with lymphocyte homing will bring us one step closer to better understanding the vast number of pathologies associated with this signaling subunit, and ultimately better the scientific understanding of lymphocyte homing dynamics in inflammatory processes (Silver & Hunter, 2010).

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