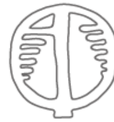


Master Thesis:

‘’ Dissecting the role of endothelial cells as atypical cancer antigen presenting cells using conditional MHCII KO mice.’’

Applicant name: Katsa Marilena

Host lab: Tsoumakidou Maria lab, Immunology division

Institute: B.S.R.C. Alexander Fleming, Vari, Athens, Greece

Date: 27 June 2019

Table of contents

.... 1
Abstract 3
Acknowledgements 5
1. **Introduction** 6
1.1 Immune system: Innate and adaptive immunity 7
1.1.1 Innate immunity 7
1.1.2 Adaptive immunity 8
1.1.2.1 Cells of Adaptive immunity 8
1.2 Antigen presentation 9
1.2.1 Professional Antigen presentation 9
1.2.1.1 Cancer-Immunity Cycle 10
1.2.2 Non-professional (Atypical) APCs 12
1.3 Tumor associated endothelial cells 13
1.3.1 Endothelial cells' role in neoangiogenesis 13
1.3.2 Endothelial cells as atypical antigen presenting cells 16
1.3.3 Tumor associated endothelial cells as atypical antigen presenting cells in TME 19
1.4 Mouse strains 21
1.4.1 Cdh5(PAC)-CreERT2: B6-Tg(Cdh5-cre/ERT2)1Rha mice 21
1.4.2 I-AB-flox : B6.129X1-H2-Ab1tm1Koni/J mice 23
2. **Materials and Methods** 25
3. **Results** 34
4. **Discussion** 40
References 44

Abstract

During this project, we were focused on **lung cancer** which is the leading cause of cancer worldwide amond men and women and also the main focus of our lab.

Tumors are complex, disorganized and chaotic, where cancer cells co-exist and co-evolve with their **stroma**. **Endothelial** cells are the most important stromal cells that play a crucial role in cancer development and seem very good candidates for the elimination of cancer. The literature analysis points out that the **immune system** is a very promising field of investigation regarding prognosis, mostly because the **stromal** microenvironment in the tumor can provide some information about what can succeed in the future concerning treatment choices and perspectives. Although the number of studies discussing lung cancer is vast, treatment efficacy is still suboptimal due to the wide range of factors that affect patient outcome.

Tumor-associated endothelial cells or tumor endothelial cells (**TECs**) refers to cells lining the tumor-associated blood vessels that control the passage of nutrients into surrounding tumor tissue.[1] They can influence the host's immune response by controlling the penetration of immune cells into the tumor and by modulating their activity. Impaired T cell transmigration is the principal and most characterized TEC-mediated mechanism that regulates immune response. More specifically, TECs crosstalk with immune cells and cancer cells through paracrine signaling but that is not totally clear.

So our main scientific question was if intratumoral endothelial cells present MHCII-cancer antigens complexes and which is the biological significance of this phenomenon in cancer.

With all the above in mind, novel **mice** strains, had to be developed, with targeted deletion of MHCII in TECs in order to elucidate their role in cancer-antigen presentation and T cell tumor tolerance. Some preliminary data showed that TECs constitute the vast majority of **MHCII**-expressing non-haemopoietic cells in the lung TME. TECs in the lung express high **MHCII** and **co-inhibitory** (PDL1) but not **co-stimulatory** molecules (CD80, CD40, CD86) being our first indicator that they play an important role as **atypical APCs**. More specifically, the models used during this thesis were **Cdh5 (PAC)-CreERT2** mice and **I-AB-flox**. As a result, their offspring had a deletion of MHCII in the endothelial cells of lung cancer.

During this project, we tried to successfully reprogram human TECs to shut down their tolerogenic functions in **cancer** and restore anti-tumor T cell responses in order to induce tumor immune rejection with minimal concerns for serious side effects.

We propose that TECs capture cancer-associated antigens and present MHCII-restricted cancer peptides in order to inhibit adaptive immune resistance. This highlights its **immunotherapeutic** targeting.

Keywords: Endothelial Cells (ECs), Tumor Associated Endothelial Cells (TECs), Tumor Microenvironment (TME), tumor, lung cancer (LC), antigen presentation, Antigen Presenting cells (APCs), professional APCs, Atypical APCs, MHCII, FACs, mouse model

Acknowledgements

Finalizing this thesis, I would like to express my sincere thanks to my **supervisor**, Dr. Tsoumakidou Maria for giving me the opportunity to be part of her research group.
By being a member of Prof. Tsoumakidou lab during my master thesis, I had the opportunity to work with lung cancer in mouse models and discover that this really fascinates me. Furthermore, I had the opportunity to learn and fulfil so many different tasks independently and I'm glad about that.
Most of all, I would like to thank my **lab mate** Dr. Kerdidani Dimitra for all of her help and support during the development of this work. I'm grateful for her guidance through this year.
Additionally, I would like to thank the members of my **Thesis Committee:** Prof. George Kollias, Dr. Vasiliki Kostourou and Dr. Vassilis Aidinis.
Of course, I would like to thank all the **lab members** for encouragement, enthusiasm and for making my everyday even better.
In the end, I would like to thank my **family** for supporting me all these years.

1. Introduction

The American Society's estimates that in the United States (2018) are about 234,030 new cases of lung cancer (121,680 in men and 112,350 in women) and about 154,050 deaths from lung cancer (83,550 in men and 70,500 in women). Lung cancer (LC) is by far the leading cause of cancer death among both men and women. LC is a disease with a poor prognosis once diagnosed, making it a very aggressive cancer with high mortality. Each year, more people die of lung cancer than of colon, breast, and prostate cancers combined. [2] For that reason there is a great need for researchers to focus on this type of cancer.

1.1 Immune system: Innate and adaptive immunity

Over the past decade, there have been numerous advances in our current understanding of the immune system and how it functions to protect the body from infection or even cancer.

As about the origin of the cells of the immune system, it's really important to mention pluripotent Hematopoietic Stem Cells (HSCs) (Fig.1).

They can differentiate in bone marrow into common lymphoid or common myeloid progenitor cells. Lymphoid stem cells give rise to B cell, T cell, and natural killer cell (NK) lineages. Myeloid stem cells give rise to a second level of lineage specific colony form unit (CFU) cells that produce granulocytes, monocytes/**macrophages**, mast cells, megakaryocytes and **erythrocytes**. Neutrophils, eosinophils and basophils are known as **granulocytes** [3]

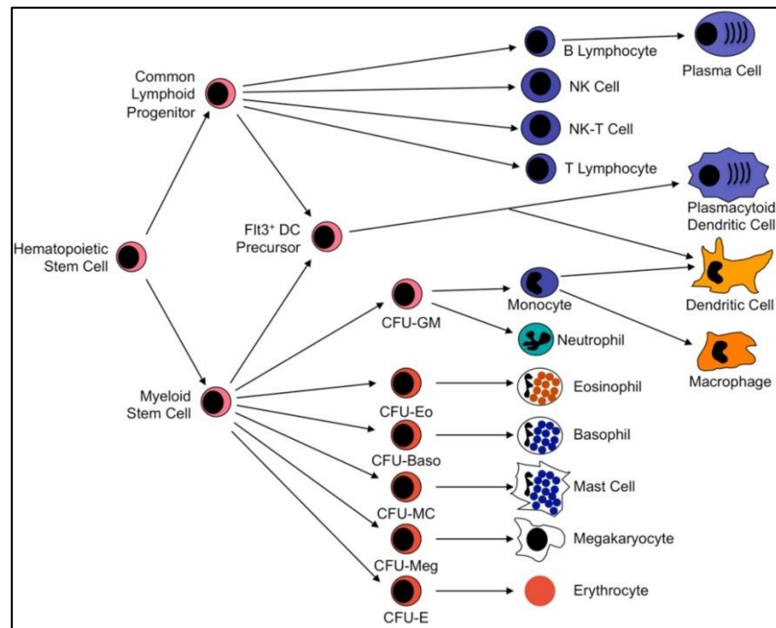


Fig.1 Hematopoietic Stem Cell-Derived Cell Lineages [4]

As already mentioned, immune system is able to destroy cancer cells and inhibit tumor growth through responses elicited by its innate and adaptive arms.[5] Innate and adaptive immunity are not mutually exclusive mechanisms of host defense, but rather are complementary, with defects in either system resulting in host vulnerability [6,7]

1.1.1 Innate immunity

Innate immunity is known as the first line of defense to an intruding pathogen. Innate immune responses are antigen nonspecific, develop quickly as they are able to appears immediately in the host or within hours of encountering an antigen. They are mediated by

various effector cells (natural killer [NK] cells, polymorphonuclear leukocytes, and mast cells, as well as antigen-presenting cells [APCs] such as macrophages and dendritic cells [DCs]), which lead to the secretion of interferon gamma (IFN-γ) and perforin, as well as inflammatory cytokines, that induce apoptosis of tumor cells.[8]
Of note, some of the cells involved in innate immunity, such as DCs, macrophages, and NK cells, also play a role in adaptive immunity.[8]

1.1.2 Adaptive immunity

During this project, we will be focused in **adaptive** immunity. More specifically, as already mentioned above, this type of immunity develops when innate immunity is ineffective in eliminating infectious agents and the infection is established. The primary functions of the adaptive immune response are the:

1. recognition of specific “non-self” antigens in the presence of “self” antigens
2. generation of pathogen-specific immunologic effector pathways that eliminate specific pathogens or pathogen infected cells
3. development of an immunologic memory that can quickly eliminate a specific pathogen should subsequent infections occur [6].

More specifically, adaptive immunity, really depends on the antigen and therefore there is a between exposure to the antigen and maximal response. Memory is the hallmark of adaptive immunity which enables the host to have a more rapid and efficient immune response upon exposure to an antigen. It comprise both humoral and cellular immunity mediated by B and T cells, respectively.[8]
In this respect, **adaptive** rather than innate immunity offers the greatest potential for durable, robust anticancer immune responses.

1.1.2.1 Cells of Adaptive immunity

As about the cells of the adaptive immune system, they include **T cells**, which are activated through the action of antigen presenting cells (**APCs**) and **B cells**.
As about **T cells**, as already mentioned, they derive from hematopoietic stem cells in bone marrow, they migrate and mature in the thymus. These cells express a unique antigen-binding receptor on their membrane, known as the T-cell receptor (TCR), and require the action of APCs (usually dendritic cells, but also macrophages, B cells, fibroblasts, endothelial and epithelial cells) to recognize a specific antigen. The surfaces of APCs express on the other hand cell-surface proteins known as the major histocompatibility complex (**MHC**).
All nucleated cells have MHC class I (also termed human leukocyte antigen [HLA] A, B and C) whereas only certain cells of the immune system (including macrophages, dendritic cells and B cells) have on their surface MHC class II (also termed HLA, DP, DQ and DR). MHCI molecules present endogenous (intracellular) peptides while MHCII molecules

present exogenous (extracellular) peptides. The MHC protein displays fragments of antigens (peptides) when a cell is infected with a pathogen or has phagocytosed foreign proteins [6]. T cells are activated when they encounter an APC that has digested an antigen and is displaying antigen fragments bound to its MHC molecules. The MHC-antigen complex activates the TCR and the T cell secretes cytokines which further control the immune response. This antigen presentation process stimulates T cells to differentiate into either cytotoxic T cells ($CD8^+$ cells) or T helper (Th) cells ($CD4^+$ cells).

Cytotoxic T (CD8) cells are primarily involved in the destruction of cells infected by foreign agents. They are activated by the interaction of their TCR with peptide-bound MHC class I molecules. Upon resolution of the infection most effector cells die and are cleared by phagocytes. However, a few of these cells are retained as memory cells that can quickly differentiate into effector cells upon subsequent encounters with the same antigen [6].

T helper (CD4) cells play an important role in establishing and maximizing the immune response. These cells have no cytotoxic or phagocytic activity, and cannot kill infected cells or clear pathogens. However, they “mediate” the immune response by directing other cells to perform these tasks.

A third type of T cell, known as the **regulatory T cell** (Treg), also plays a role in the immune response. Treg cells limit and suppress the immune system and, thereby, may function to control aberrant immune responses to self-antigens and the development of autoimmune disease. [6] With unique capacity for immune inhibition, they can impair anti-tumour immunity and help tumor cells to escape from immune surveillance.[9]

1.2 Antigen presentation

1.2.1 Professional Antigen presentation

Macrophages, dendritic cells and B lymphocytes are characterized as the professional Antigen Presenting Cells (APCs). Although all cells can present peptides via MHCI to $CD8^+$ T cells, only professional APCs can present peptides via MHCI as well as MHCII to $CD8^+$ or $CD4^+$ T cells. Professional APCs can also provide co-stimulatory molecules and instructive cytokines, which are all necessary for induction of primary T cell responses.

For an adaptive immune response to be effective a second cell is required in order to select and expand those few T cell clones that express ‘useful’ antigen receptors. This quality was termed ‘immunogenicity’ [10], and requires ‘immune response’ genes, which map to the MHC locus [11,12]. Immunogenicity requires that proteins are processed and presented on MHC molecules to be recognized by T cells. However, in order for T cells to be activated more than the simple, expression of MHC molecules by antigen-presenting cells (APCs) is necessary.

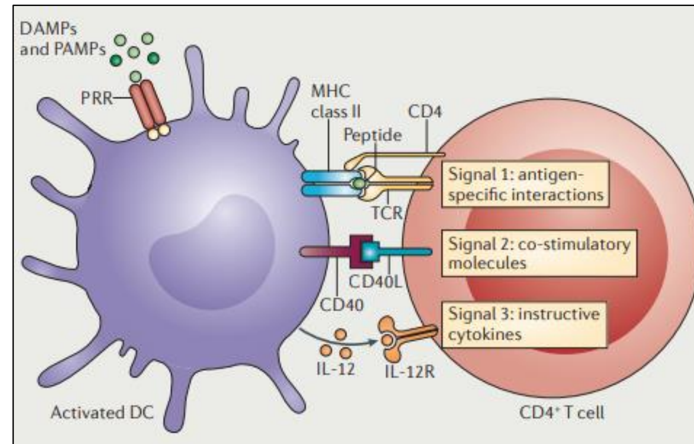


Fig.2 Antigen presentation. [13]

The activation of a naive T cell requires interaction with an APC that provides multiple signals. Induction of a T cell response requires three canonical signals to be provided by the APC: Signal 1' is delivered through interaction of the TCR-peptide complex with MHC (MHCI or MHCII, both phagocytose and process Ag). Moreover, signal 2' involves cell surface co-stimulatory molecules (f.e CD40-CD40L) whereas signal 3' is mediated by instructive secreted cytokines.(Fig.2) The ability to deliver these three signals is the defining characteristic of a professional APC [14]. And the appropriate combination of all these three inputs is required for the activation of naive T cells. The quality of each of these signals determines the strength and type of responses generated.[15,16] The search to identify APCs focused on cells that could induce B cell and T cell responses in vitro and in vivo.

1.2.1.1 Cancer-Immunity Cycle

The generation of immunity to cancer is a cyclic process that can be self-propagating, leading to an accumulation of immune-stimulatory factors that in principle should amplify and broaden T cell responses. This cycle is also characterized by inhibitory factors that lead to immune regulatory feedback mechanisms, which can halt the development or limit the immunity.

In the first step (Fig.3), oncogenesis creates neoantigens that are released and captured by dendritic cells (DCs) for processing (step 1). In order for this step to lead to an anticancer T cell response, it must come along with signals that specify immunity in order not peripheral tolerance to be induced to tumor antigens. Next, DCs present the captured antigens on MHCI and MHCII molecules to T cells (step 2), resulting in the priming and activation of effector T cell responses against the cancer-specific antigens (step 3) that are viewed as foreign or against which central tolerance has been incomplete. The nature of the immune response is determined at this stage, with a critical balance representing the ratio of T effector cells versus **Treg** cells being key factors for the final outcome. Tregs are

able to limit damage to nontumor self-antigens. Finally, the activated effector T cells traffic to (step 4) and infiltrate the tumor bed (step 5), specifically recognize and bind to cancer cells through the interaction between its T cell receptor (TCR) and its cognate antigen bound to MHCI (step 6), and kill their target cancer cell (step 7). Killing of the cancer cell releases additional tumor-associated antigens (step 1 again) to increase the breadth and depth of the response in subsequent revolutions of the cycle.

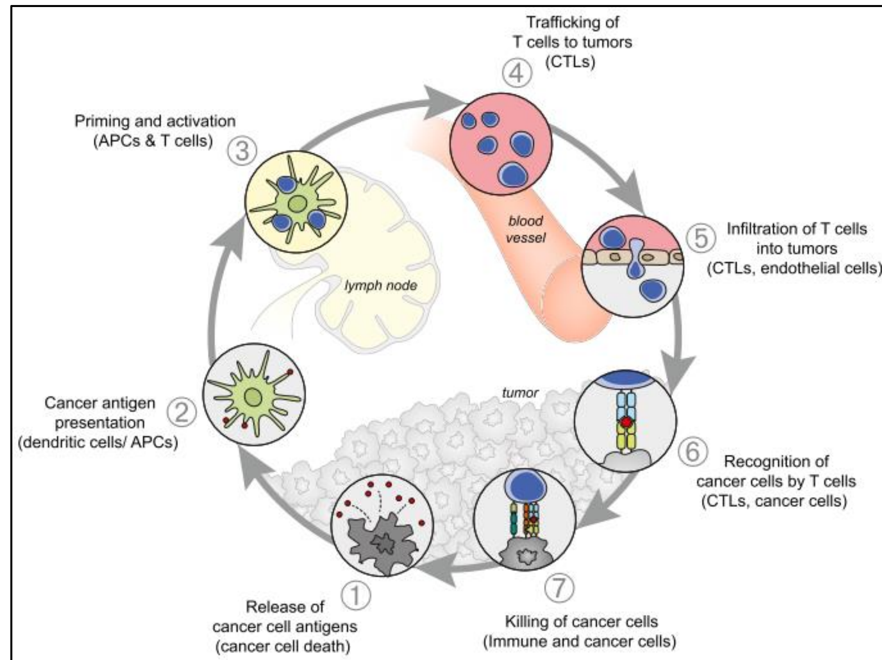


Fig.3 The Cancer-Immunity Cycle [17]

Cancer is able to alter this cancer-immunity cycle to its advantage and promote its survival. There exists a cancer-immune set point that is based upon the balance of immune stimulatory and inhibitory factors, both of which are influenced by the tumor and host genetics, and environment.[17]

As far as lung cancer cells is concern that they are particularly immunogenic, due to high nonsynonymous mutational loads. [18] The main effectors against cancer cells are **$CD8^+$ T cells**. As already mentioned, professional APCs are responsible for their initial priming and afterwards they can kill cancer cells that express MHCI-peptide complexes. $CD8^+$ T cell responses are inhibited when cancer antigen-specific $CD4^+$ T cells are missing. **$CD4^+$ T cells** are also needed in order to provide the necessary cytokine signals for sufficient CD8+ T cell expansion and acquisition of cytolytic activity [19]. Type, density, location, and organization of T cells within lung tumors are a major determinant of patient outcome.[20,21]

Taken all together in the concept of cancer, neoantigens are presented via the major histocompatibility (MHC) class I and II molecules on antigen presenting cells (APCs) and bind to the corresponding T-cell receptor (TCR). Naïve T-cells can then proliferate into effector memory T-cells, effector T-cells, or exhausted effector T-cells.[22] This response is

balanced by the presence of regulatory T-cells (**Tregs**) that limit damage to nontumor self-antigens. These activated effector T-cells are subsequently recruited into the TME resulting in the targeted killing of tumor cells. [17]

In general, **atypical antigen presentation** in cancer is a less analyzed procedure and that was the reason we focused on it.

1.2.2 Non-professional (Atypical) APCs

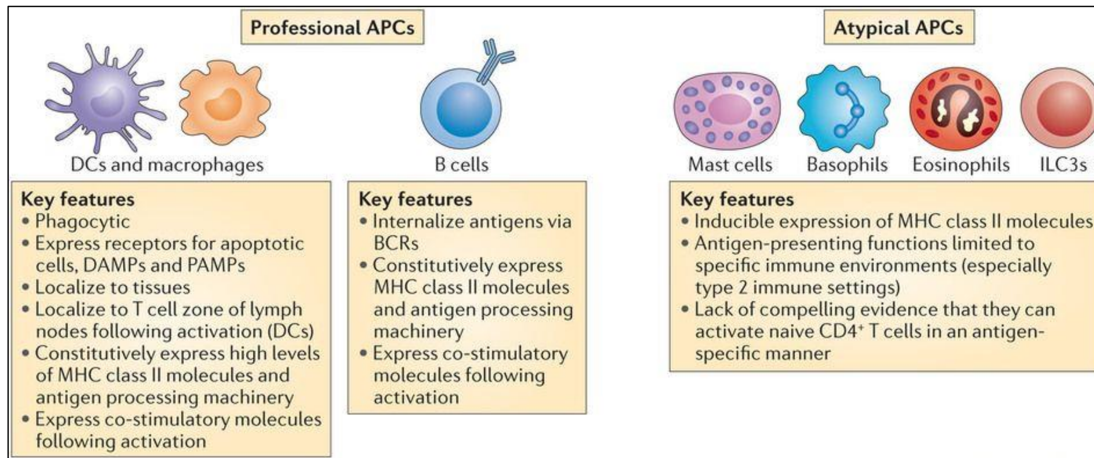


Fig.4 Key antigen-presenting functions of professional and atypical antigen-presenting cells [13]

A number of haematopoietic cell types and non-haematopoietic cell types such as: myofibroblasts, **endothelial**, epithelial and lymph node stromal cells are proposed to express MHC class II molecules. Non-professional antigen-presenting cells can be induced to express class II MHC molecules or a co-stimulatory signal. Many of these cells function in antigen presentation only for short periods of time during a sustained inflammatory response. MHC class II expression alone is not sufficient for full antigen presenting (APC) function. Thus, they rather modulate and usually suppress T cell responses that have been initiated by professional APCs [13], either through direct contact with T cells or through transfer of MHC-peptide complexes for indirect presentation by professional APCs (cross-dressing) [23]. This is particularly relevant for endothelial cells which are known to express MHCII and may communicate with other cells through extracellular vesicles (exosomes) [24,25].

We were mostly interested in the investigation of the role of **endothelial** cells as potential antigen presenting cells in the lung tumor microenvironment. Lung cancer is the main focus of our lab.

1.3 Tumor associated endothelial cells

1.3.1 Endothelial cells' role in neoangiogenesis

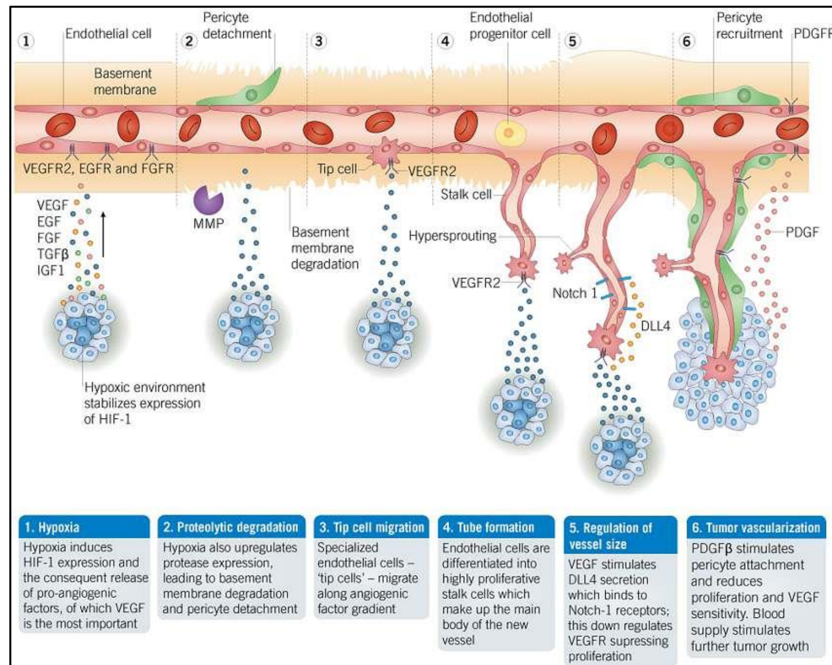


Fig.5 Angiogenesis steps in tumor vascularization (Cancer Research Product Guide Edition 3, 2015)

In embryogenesis, blood vessels are formed de novo by vasculogenesis involving bone marrow-derived endothelial progenitor cells (EPCs) [26] followed by **angiogenesis**, a process where new blood vessels are created from pre-existing vasculature.[27] Blood vessels create a network of tubes and capillaries and consist of **endothelial** cells (ECs) which create a tight barrier between the blood and tissue, and interact with extracellular matrix (ECM).

Endothelium refers to cells that line the interior surface of blood vessels and lymphatic vessels,[28] forming an interface between circulating blood or lymph in the lumen and the rest of the vessel wall. It is a thin layer of simple, or single-layered, squamous cells called endothelial cells. More specifically, endothelial cells in direct contact with blood are called vascular endothelial cells (BECs), whereas those in direct contact with lymph are known as lymphatic endothelial cells (LECs). More specifically, BECs maintain the integrity of blood vessels and ensure the supply of oxygen and other nutrients to the tissue supporting its progression.

Once activated, the endothelium produces several pro-angiogenic factors. Indeed, whenever the angiogenic switch occurs, the neo-vascular tips actively produce tumor-promoting factors such as active TGF-b1, periostin (POSTN) 65, and other angiocrine factors like adhesion molecules (ICAM 1, VCAM 1, E-selectin, P-selectin and hyaluronan) and chemokines (IL-8, MCP1 and SDF1) [29]. Such environment activates a tight coordination

between tip cells and stalk cells where migrating tip cells are followed by stalk cells, which proliferate to elongate the new sprout, form a lumen and recruit pericytes for vessel stabilization.

Tumors are developing organ-like structures with a high metabolic demand, which are, therefore, in need of a sufficient blood supply. The generation of new blood vessels is estimated to happen when tumors are developing beyond 100-200 mm [30]. Neoplastic transformation is often accompanied by the formation of a tumor bed and profound alterations in the surrounding connective tissue and stroma, a process that culminates in the establishment of a pathological tumor microenvironment (TME). Apart from cancer cells, the TME of a solid tumor contains a complex interstitial extracellular matrix and various stromal cells that are recruited from the surrounding tissues or from the bone marrow and include fibroblasts, cells of the immune systems, pericytes, and endothelial cells (ECs) of the blood and lymphatic vasculature. [31]

Structural abnormalities of the underlying stroma have been associated with the irregular features of the tumor vasculature. Tumor blood vessels are responsible for removing waste products from tumor tissue and providing a getaway for tumor metastasis. The vessels in tumor have chaotic blood flow and can become leaky due to loose endothelial cell interconnections. [32] BECs in cancer lesions are irregularly shaped and form luminal projections. [33]

Increased interstitial pressure and inadequate drainage by the lymphatic vessels is also observed. The high interstitial fluid pressure in a tumor causes blood vessel collapse and impedes blood flow. This is one reason why tumor tissue is usually under a **hypoxic** condition, even though it is highly vascularized. Abnormal angiogenesis is a feature of pathological conditions including tumor progression, where hyperproliferating cancer cells surpass their blood supply and become hypoxic. Hypoxia often results in the excessive production of angiogenic factors by multiple stromal cells (Fig.5). It is considered to be the main trigger of tumor neoangiogenesis, which encompasses sprouting new vasculature from already established vessels, a process guided by proliferation of endothelial cells towards a gradient of pro-angiogenic signals.

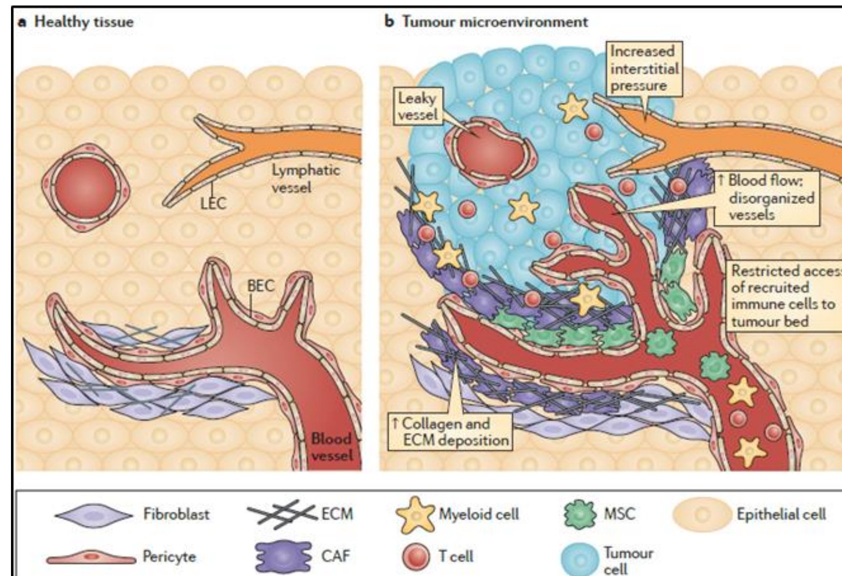


Fig .6 Cellular and architectural changes in the tumor microenvironment [34]

Finally, various chemokines and cytokines in the TME (Fig.6) attract activated T cells and myeloid cells to the tumor lesion, but tortuous blood vessels and dense ECM often hinder their access to the tumor nest. Although the makeup of the cellular and extracellular milieu can differ between tumor types and stages of growth, it is becoming clear that changes in the cellular architecture of the TME can influence tumor growth, metastasis and drug resistance.

As already mentioned above, tumor have a higher metabolic demand. To be more specific, metabolism is altered in TME, mainly through expression of hypoxia-dependent expression of glycolytic enzymes as well as the secretion of pro-angiogenic/pro-glycolytic growth factors. In fact, TECs display higher rates of glycolysis than normal endothelial cells in healthy tissues [35]. Moreover, lactate accumulation in the hypoxic tumor environment can result in lactate uptake by TEC which induces reactive oxygen species (ROS)-mediated pro-inflammatory [36] activation of VEGF2 and promotion of angiogenesis [37].

Another important feature of TECs is that they can play a role in tumor restraint. Experimental evidence showed that implantation of endothelial cells embedded in porous matrices slowed the growth of adjacent tumor xenograft (Baker et al. 2011). Indeed, conditioned medium from quiescent endothelial cells altered the proliferative or invasive capacity of lung cancer cells in vitro and reduced pro- tumorigenic and pro-inflammatory signals in cancer cells (Baker at al. 2011). As an example, Perlecan, the major extra-cellular heparan sulfate proteoglycan expressed by endothelial cells, functions as a master regulator of vascular biology. For instance, silencing of Perlecan in endothelial cells abolished their ability to inhibit cancer-cell invasiveness, possibly because due to increased secretion of IL-6 (Baker et al. 2011). Moreover, contact-dependent interactions

between cancer cell receptor (KAI1) and TEC surface receptor (DARC) can favor carcinoma senescence and reduce metastasis [39].

Endothelial cells, as basic components of blood vessels seem very promising candidates, for anti-angiogenic and potentially anti-tumor therapy. Some reasons are mentioned above:

i. one tumor endothelial cell can support a lot of tumor cells, thus, targeting endothelial cells might be an even more effective strategy than targeting tumor cells
ii. tumor endothelial cells are the same among all tumor types, thus, an anti-angiogenic drug could be ideally useful in treating all cancers
iii. until recently, tumor endothelial cells have been believed to be genetically stable, so they might not acquire drug resistance, unlike tumor cells. However, recent studies suggest that tumor endothelial cells might be different from normal endothelial cells and might also be heterogeneous among organs or tumor types. Furthermore, the therapeutic efficacy of anti-angiogenic therapy is not dramatic but rather marginal.[40]

Based on the findings that the disorganized nature of tumor blood vessels is also an obstacle to tumor infiltrating lymphocytes, it is possible that similar approaches might enhance antitumor immunity. [41] For that reasons, targeting tumor endothelial cells that are the most important cells for angiogenesis can help tumor elimination. Endothelial cells are even more important cells because there is evidence that can interact with the immune system.

1.3.2 Endothelial cells as atypical antigen presenting cells

The capacity of cells to form and display peptide antigens complexed to MHC molecules, class I to $CD8^+$ T lymphocytes and class II to $CD4^+$ T lymphocytes, is a prerequisite for **antigen presentation**. In comparison with the professional antigen presenting cells that can provide 3 signals, endothelial are equipped with all of the necessary capabilities, with one key exception, to provide APC signals 1-3 already mentioned in the professional antigen presenting cells. [42]

Besides the canonical 'professional' Ag Presenting Cells, **endothelial cells** are one of the few cell types that express both Major Histocompatibility Complex (MHC)-I and –II along with co-stimulatory molecules, which are necessary to present peptide Ag to $CD8^+$ and $CD4^+$ T cells.

Peptide antigens presented by ECs may be self-derived, though the same peptide-MHC complex may be tolerogenic or pro-inflammatory in response to the milieu in which lymphocyte encounter occurs.[43] Microvascular ECs in humans and most other mammals basally express both class I and class II MHC molecules *in vivo*[44]. More specifically, it's shown in human allografts that they can express both MHC class I and class II molecules,

and can present processed protein antigens to T cell clones [45–47]. However, the abundance of MHC expression by ECs differs among vascular segments and can vary in response to environmental signals[48].

It is also shown that the endothelium can activate previously primed (i.e effector, memory) CD8$^+$ and CD4$^+$ T cells (but not naïve T cells) in an Ag-specific manner, through a novel ILP (invadopodia like protrusions) enriched immunological synapse (a 'Podo-Synapse'). Thus the endothelium may play unique roles as ancillary or 'semi-professional' APCs that function during the effector phase of immune responses. (Fig.9)

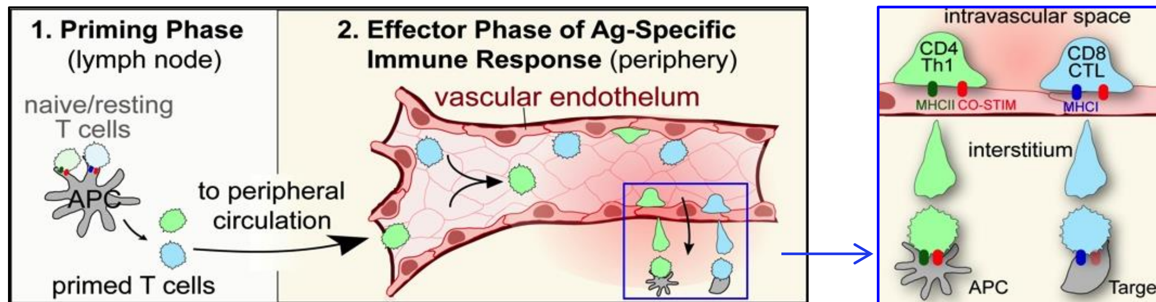


Fig. 9 Role of endothelial cells as antigen presenting cells (Carman lab, Harvard school)

This immune regulatory function is well studied for Lymph Node (LN) resident LECs. [49] Lymphatic endothelial cells have a high endocytic capacity and within lymphoid organs are able to present exogenous antigen to T-cells on both MHC-I and MHC-II molecules and modulate their activation status [50–52]. LEC express MHC-I and MHC-II, and present antigen on MHC-I via both direct and cross-presentation[53]. LECs endocytose and cross-present MHC-I antigens, although they do it less efficiently than professional APC.[54] However, LEC do not express co-stimulatory molecules CD80, CD86, 4-1BBL, or OX40L extracellularly or intracellularly[53]. Additionally, they represent a stromal cell population expressing peripheral tissue restricted antigens and mediate the deletion of autoreactive CD8$^+$ T-cells[50,55,56]. Moreover, LECs are potent immunoregulators and can inhibit DC-mediated antigen presentation and bystander T-cell proliferation via direct contact and nitric oxide production [51,57].

Yet, constitutive expression of MHC-II was subsequently demonstrated in murine liver sinusoidal ([58];[59];[60]) and lung endothelium ([61]) and on lymphatic endothelia of LN.[60] Sage and Carman, had recently quantified variable levels of constitutive MHC-II expression in mouse heart, lung, kidney, liver, and skin with the most striking levels found on lung endothelium (unpublished observations). As about the liver, a well-known strongly tolerogenic environment, liver sinusoidal ECs (LSECs) exhibit extremely efficient scavenging, cross-presentation, and have unique tolerogenic functions. More specifically, LSECs scavenge and cross-present food-borne antigens and induce tolerance through T

cell adhesion and sequestration in the liver [58,62] and tumor-associated LECs cross-present exogenous antigens [52,54] and maintain peripheral tolerance to self-antigens in LNs [56,59].

Human EC express CD40, CD58 (LFA-3), CD134 (OX40) ligand and ICOS ligand which can contribute to T cell activation in vitro [63–65]. However, there is no evidence either in vitro or in vivo that human endothelial cells can express **CD80 or CD86,** which are necessary to stimulate naive allo-reactive T cells [45].

In mice, is proved that endothelial cells can express **Ag processing machinery** (e.g., LMP2, 7, TAP1, 2, invariant chain, and HLA-DM) and it's shown that can efficiently take up, process, and present/crosspresent Ag in vitro and in vivo [42] Moreover, endothelia express a significant range of **co-stimulatory** (e.g., ICAM-1, VCAM-1, CD40, LFA-3, ICOSL, 4-1BB, OX40L, and TL1A) and **co-inhibitory** (e.g., PD-L1 and PD-L2) molecules, as well as cytokines, both of which are regulated by inflammatory cue. More specifically, in addition to influencing T cell extravasation, models of inflammation have shown that blood endothelial cancer cells (BECs) can function as **antigen presenting** cells that display peptide-MHC complexes and express several immunoregulatory co-receptors [66,67].

Absent from most endothelia are CD80 or CD86 co-stimulators that are indispensable for the activation of naive lymphocytes.[60]

In general, TECs express antigen processing machinery, MHC class I and II, co-stimulatory and co-inhibitory molecules, suggesting that they might play an important role as **atypical APCs**.[68] There is a relative significance of EC antigen presentation in vivo, however, is likely both tissue and disease specific. Further testing is needed and specifically, EC-specific knockdown strategies, to determine the functional relevance of EC antigen-processing and presentation in vivo

1.3.3 Tumor associated endothelial cells as atypical antigen presenting cells in TME

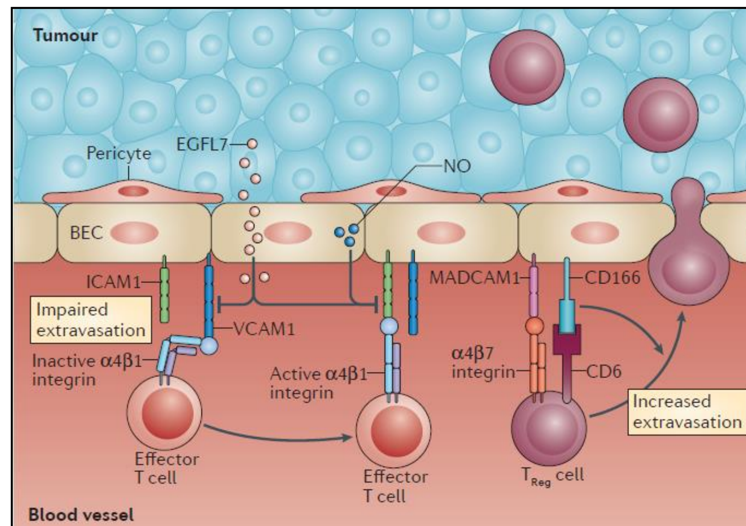


Fig.7 Regulation of immune cell infiltration into the TME [34]

The trafficking of newly activated antigen-specific T cells from the blood into the tumor bed is thought to be dysfunctional in **cancer**, and many obstacles, including stromal cells, impair this process (Fig.8). T-cell exhaustion is a state of T-cell dysfunction in chronic environment, exhausted T cells express high levels of inhibitory receptors, including programmed cell death protein 1 (**PD-1**), lymphocyte activation gene 3 protein (LAG-3), T-cell immunoglobulin domain and mucin domain protein 3 (**TIM-3**), cytotoxic T lymphocyte antigen-4 (CTLA-4), band T lymphocyte attenuator (BTLA) and T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (**TIGIT**).(DL et al., 2006; A and EJ, 2009; SD et al., 2009; AC et al., 2010; N et al., 2011; J et al., 2012)
There is now evidence that the same processes may also occur for BECs in **cancer**. BECs can express the PD1 ligands **PDL1** and **PDL2** as well as ligands for other receptors of the CD28–CTLA4 family, such as B7-H3 and B7-H4, which induce $CD8^{+}$ T cell tolerance and correlate with poor prognosis in patients with cancer [69]. PDL1 is able to inhibit T cells via activation of the PD1 receptor by inducing a state of T-cell unresponsiveness, termed T cell exhaustion that is mentioned above as well[49]. More specifically, it's shown in patients with lymphoma that BECs isolated from lymph nodes express the co-inhibitory molecule T cell immunoglobulin and mucin-containing domain molecule 3 (**TIM3**), which promoted the growth and dissemination of lymphomas by inhibiting TH1 cell polarization [70].
Blood endothelial cells (BECs), can also express CD137 [71], a glycoprotein expressed by activated T cells, particularly $CD8^{+}$ T cells, that generally functions as a co-stimulatory molecule. Interestingly, tumor BECs express a soluble form of CD137 that antagonizes membrane-bound CD137, which limits its co-stimulatory effects and thus contributes to a suppressive environment for T cells (Fig. 8).

In the context of cancer, BECs can release soluble factors that modulate T cell responses, especially upon exposure to inflammatory stimuli from the **TME**. For instance, exposure to **tumor** cells drives the secretion of PGE2 , IL-6, transforming growth factor-β (TGFβ), and VEGFA by BECs [72–74]. Furthermore, supernatant from cultured **lung tumor**-derived BECs disrupted CD3-mediated T cell activation in vitro [73]. Finally, BECs from patients with **renal cell carcinoma**, high-grade **tumors,** have been shown to express the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO)[75].

Platelet endothelial cell adhesion molecule 1 (PECAM1 or CD31) has been implicated in immune escape of **tumors**. As PECAM1 is highly expressed by endothelial cells, it is likely that BECs in the TME may also promote T cell tolerance through the regulation of TCR signaling. In addition to co-stimulatory ligands, tumor BECs can express molecules that induce apoptosis of cytotoxic lymphocytes, including tumor necrosis factor (TNF)- related apoptosis inducing ligand (TRAIL) and CD95 ligand (FasL) (Gregory T. Motz, et al. 2014).

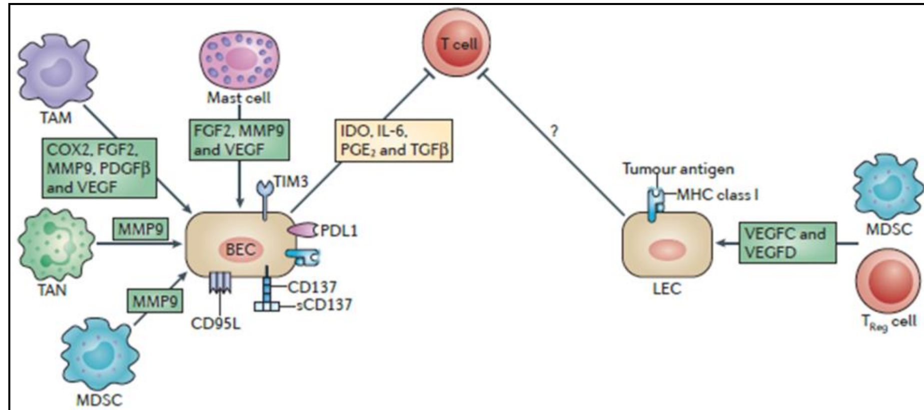


Fig.8 BEC & LEC crosstalk with immune cell in the TME [34]

Peripheral tissue antigens or **tumor** antigens may be transferred from LECs to dendritic cells, which present these antigens to $CD4^{+}$ T cells in the absence of co-stimulatory molecules, thereby inducing anergy. In tumors, LECs are also capable of scavenging tumor-associated antigens and cross-presenting them on MHC-I [77], however, whether LEC antigen presentation functionally contributes to peripheral T cell responses remains unknown. Stimulation of LECs by VEGF-C and inflammatory cytokines TNFα and IFNγ can reduce CD86 expression on dendritic cells and produce IDO, which depletes tryptophan from the microenvironment, thereby preventing the activation of T cells. [78] Presentation of tumor antigen by peripheral and LN LECs, was observed suggesting that LECs may contribute directly to the inhibition of T-cell-mediated antitumor immune responses [77]. However, the mechanisms behind the T-cell inhibition by tumor-associated LECs have not been investigated so far.

Collectively, these studies suggest an important role for tumor vasculature in modulating **antitumor** immunity. Given that BECs and LECs in the TME are thought to dampen T cell

infiltration and/or activity, targeting pro-angiogenic and pro-lymphangiogenic pathways could hold promise for enhancing antitumor immunity, in particular when combined with other immunotherapeutic approaches. All these findings on the multifaceted role of Tumor-associated endothelial cells in cancers' growth and progression underline the need for further characterization of TEC interaction in tumor stroma in the context of cancer. This is expected to open new perspectives for cancer treatment and shed new light on the diatribe between antiangiogenesis and vascular normalization approaches.

1.4 Mouse strains

Transgenic mice are ideal for investigating antigen presentation by particular cell types in vivo. The mouse models used during this thesis were **Cdh5(PAC)-CreERT2** mice and **I-AB-flox** mice and are presented below.

1.4.1 Cdh5(PAC)-CreERT2: B6-Tg(Cdh5-cre/ERT2)1Rha mice

As about Cdh5(PAC)-CreERT2 mouse, it was developed in the laboratory of Ralf Adams at the London Research Institute. The model was generated by microinjecting a transgene containing a genomic Cdh5(PAC) promoter fragment fused to a CreERT2 cDNA into C57BL/6 zygotes. [79] Founder lines were backcrossed to C57BL/6 for at least five generations.

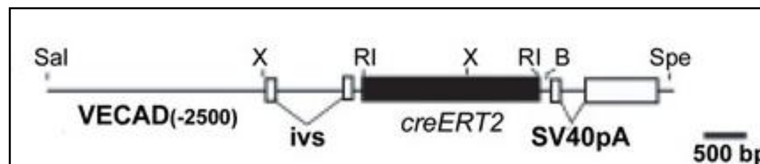


Fig.10 cDNA encoding the tamoxifen-sensitive Cre-ERT2 was subcloned downstream of the murine VE-cadherin endothelial-specific promoter [79]

More specifically, to introduce temporal control in genetic experiments targeting the endothelium, a mouse line expressing tamoxifen-inducible Cre-recombinase (CreERT2) was established under the regulation of the vascular endothelial cadherin promoter (VECAD). Specificity and efficiency of Cre activity was documented by crossing VECad-Cre-ERT2 with the ROSA26R reporter mouse, in which a floxed-stop cassette was placed upstream of the-galactosidase gene.

In order to understand the role of a given gene product in a given cell type at a given developmental stage, genetic techniques have been developed that allow for the introduction of defined mutations into the mouse genome at will, in a specific cell type and at a chosen time [80,81] Most current conditional gene-targeting systems are based on

the use of the site-specific recombinase Cre (cyclization recombination) which catalyzes recombination between two 34-bp DNA recognition sites named loxP. The basic strategy for Cre/lox-directed gene knockout experiments is to flank, or ‘‘flox’’, an essential exon of the gene of interest with two loxP sites (by homologous recombination in embryonic stem cells), and then to ‘‘deliver’’ Cre that excises the intervening DNA including the exon from the chromosome, thus generating a null allele in all cells where Cre is active. Delivery of Cre can be achieved by crossing mice carrying the floxed target gene with transgenic Cre-expressing mice.

To add inducibility to the Cre/lox system, ligand-dependent chimeric Cre recombinases, so-called CreER recombinases, have been developed [82–85]They consist of Cre fused to mutated hormone-binding domains of the estrogen receptor. The CreER recombinases are inactive, but can be activated by the synthetic estrogen receptor ligand 4-hydroxytamoxifen (OHT), therefore allowing for external temporal control of Cre activity.

Indeed, by combining tissue-specific expression of a CreER recombinase with its tamoxifen-dependent activity, the excision of floxed chromosomal DNA can be controlled both spatially and temporally by treating the mouse with tamoxifen, which is metabolized to OHT. The operating mode of inducible CreER mice is outlined in Fig. 10.

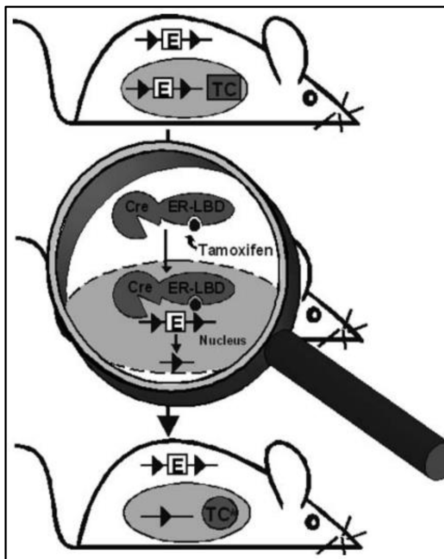


Fig.**11** Inducible Cre Mice

The CreERT2 recombinase, which contains the human estrogen receptor ligand-binding domain with a G400V/M543A/L544A triple mutation, is currently the sharpest tool in the CreER box and its use is highly recommended for inducible mutagenesis in the mouse [83,86,87]). A convenient way to characterize the recombination properties of a given Cre transgenic mouse line is the use of Cre reporter mice, the most popular one being the so-called R26R line that produces -galactosidase after Cre-mediated excision of a STOP cassette from the broadly expressed ROSA26 locus [88].

The following sections describe the generation and analysis of inducible mouse mutants using CreERT2 transgenic mice and R26R reporter mice [89]

The transgene contains a splice acceptor sequence (SA) upstream of a neomycin gene (neo) flanked by lox P sites and downstream of a LacZ gene. Following Cre recombination, the neo gene is removed and the LacZ gene is constitutively expressed by the ROSA26 promoter (Fig.12).

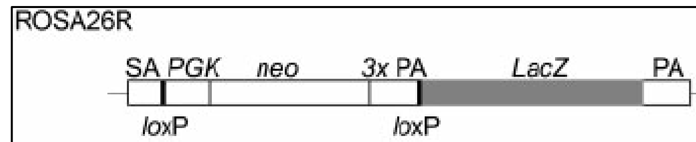


Fig.12 Structure of the ROSA26 locus in Cre reporter mice [90]

Tamoxifen was found that specifically induce widespread recombination in the endothelium of embryonic, neonatal, and adult tissues. Recombination was also documented in tumor-associated vascular beds and in postnatal angiogenesis assays. Furthermore, injection of tamoxifen in adult animals resulted in negligible excision (lower than 0.4%) in the hematopoietic lineage. The **VECad-Cre-ERT2** mouse is likely to be a valuable tool to study the function of genes involved in vascular development, homeostasis, and in complex processes involving neoangiogenesis, such as tumor growth.
This mouse carries a transgene with tamoxifen inducible Cre recombinase, generating vascular endothelial specific targeted mutants.[91]
VE-Cad-Cre-ERT2 mice were crossed with **IAb$^{f/f}$** mice to generate homozygous **CreIAb$^{f/f}$** mice. Wildtype IAb$^{f/f}$ mice served as controls in all experiments.

1.4.2 I-AB-flox : B6.129X1-H2-Ab1tm1Koni/J mice

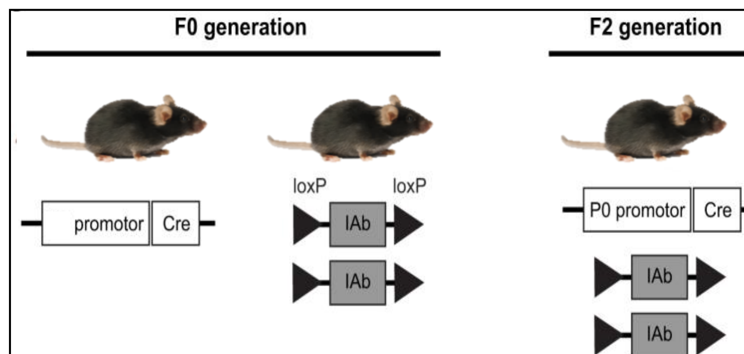


Fig.13 Cre IAb$^{f/f}$ mouse [92]

As about I-AB-flox mice, it possesses a loxP-flanked cassette upstream and a loxP site downstream of exon 1 of the MHCII H2-Ab1 locus. When bred with mice expressing Cre-recombinase driven by a specific promoter, iab deficiency results in the loss of MHCII from the surface the Cre-recombinase targeted cells.

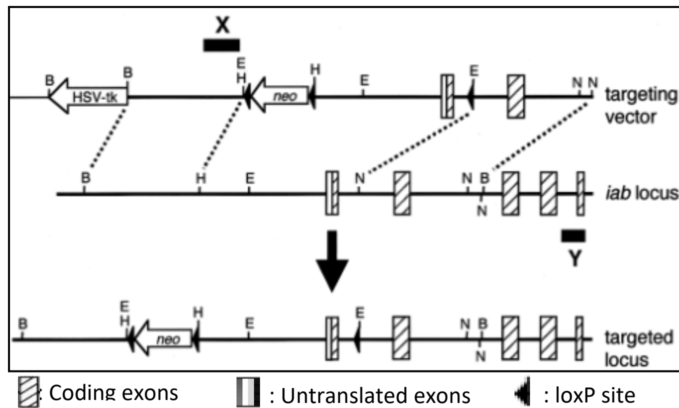


Fig.15 Generation of a conditional iab allele.[92]

Regarding the figure on the left, the empty and hatched boxes represent the untranslated and coding exons, respectively. The loxP sites are depicted as filled arrow heads. Bars mark the positions of probes X and Y. Probe X is a HindIII/SacI region from the genomic clone.

Probe Y is an EcoRI-excisable PCR clone generated using the well-defined iab genomic sequence. Endonuclease sites shown are BamHI (B),EcoRV (E),HindIII (H), andNdeI (N). Homologous recombinants were generated in 129/SvJ embryonic stem cells (Incyte). Chimeric mice were bred with C57BL/6Jmice.[92]

2. Materials and Methods

Preliminary experiment

Firstly, some preliminary experiments had to be done. Syngeneic immunocompetent mice were used. Two independent experiments were completed with 2 mice per group. Lung orthotopic transplantation of LLC-OVA cells was done in 2 months old mice and the mice were sacrificed after 2 weeks. (The whole procedure is explained in more details in experiments below)

Total tumor cells were stained for MHCII, haemopoietic (**CD45**), helper T lymphocytes (**CD4**), cytotoxic-T lymphocytes (**CD8**) and endothelial (**CD31**) markers and analyzed by FACS. Except CD4, CD8 and CD31, the rest markers were used to exclude the corresponding populations.

Briefly, lung tumors and tumor-free lung tissue were enzymatically digested and stained with a dead cell dye and a 17 antibody panel focused on stromal- associated and immune markers (antigen-presentation, co-stimulatory/inhibitory molecules, cytokines and chemokines). A set of lineage-imprinted markers were used to roughly subdivide cells into endothelial cells. The additional markers enabled the characterization of the heterogeneity of the cells and were able to identify cancer-induced subsets with potential tolerogenic antigen-presenting functions.

It was the first indicator that endothelial cells play a role as atypical antigen presenting cells in lung cancer and gave us the confidence to start.

Experiments' Matings

The **Cdh5 (PAC)-CreERT2 (Cdh5CRE +/-)** mice was crossed to **I-AB-flox (MHCII f/f) mice**, that are described above. The offsprings were Cdh5CRE+MHCIIF/F or Cdh5CRE- MHCIIF/F. All mice strains to be used were on Bl6 background and were expressing the I-AB MHCII gene. The time needed for the matings was approximately 5 months. During the whole period, there was a need for genotyping with Polymerase chain reactions (PCRs).

Extraction

Before that, we had to do extraction of DNA in order to use it for PCR. A small piece of tail of 7-12days' mice was cut. If the mice were older, a small piece of ear was cut, alternatively. Each piece of tissue (tail or ear), was put in a tube with a mix of 400µl tail buffer with 4µl proteinase K and was let in 55°c overnight (8-72h) in the incubator.

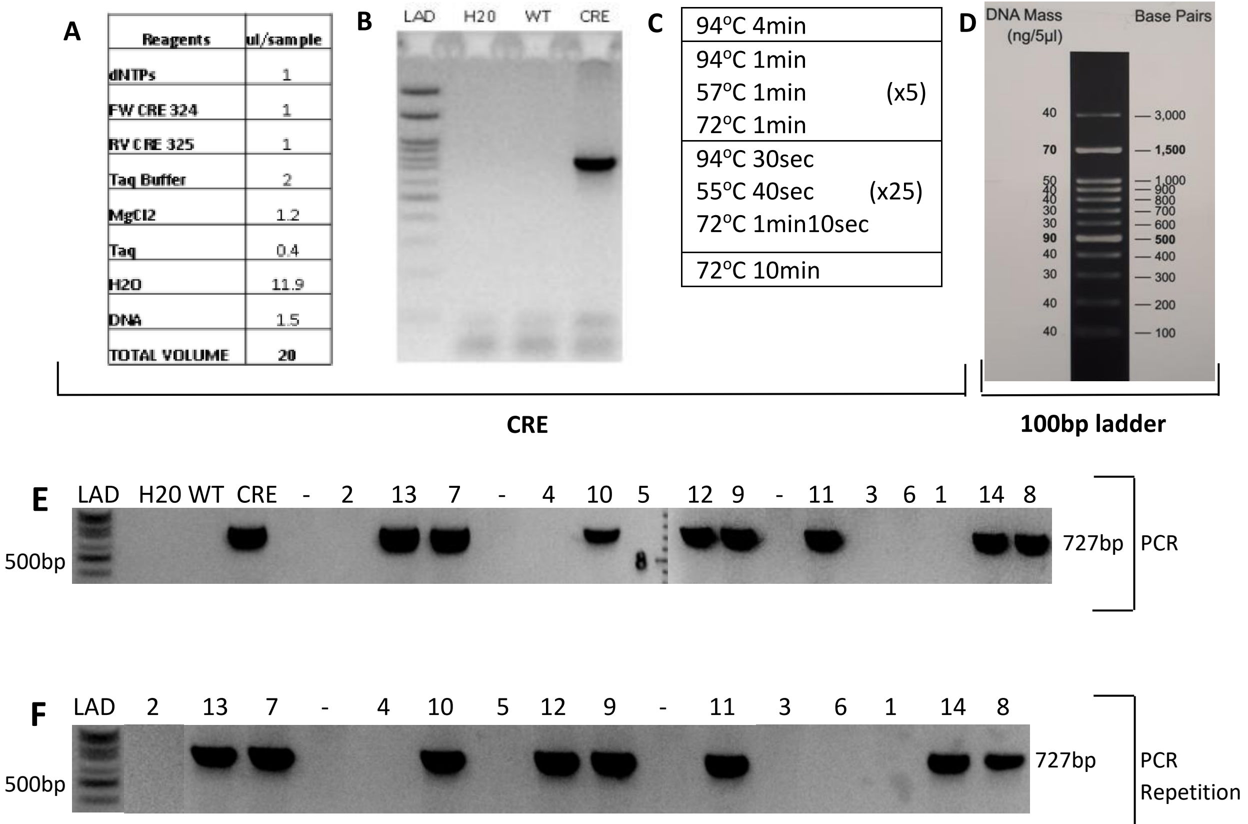
The second day, the following protocol was followed. Firstly, we checked if the samples were well dissolved and we did vortex them. 400µl of phenol were added and vortex was followed for 1-2min. Then, spin was done at 12000 rpm for 10min and new tubes were labeled. 300µl of upper DNA (aqueous phase) were removed in the new tubes, 300µl of

isopropanol were added and well shaking was done. DNA was fished with glass pasteur pipette, pipette was dipped in 70% and then in 100% ethanol and was left upside-down to dry. Afterwards, they were placed it in 200µl H20 and were left to dissolve. In the end, the tubes were stored in the cold room till used for PCR

Polymerase chain reaction (PCR)

For each **Cdh5a CRE+/- MHCIIf/f** offspring with **I-AB-flox (MHCIIf/f)** mouse we had to do PCR in order to check the genotyping. The offsprings (experimental mice) were already MHCIIf/f, because both parents were, so only genotyping for CRE had to be done. We did 2 repetitions of **CRE PCR** in order to validate our results. A master mix was made including dNTPs, primers, Taq buffer, $MgCl_2$, Taq and water, and then each DNA was added in the correct amount based on Fig16A. As about the primers used, they were: FW CRE 324 (Invitrogen by Thermo Scientific, 645762V9668, D07) and RV CRE 325 (Invitrogen by Thermo Scientific, 848409R6200, E03). The tubes including all the PCR ingredients, were put in a PCR machine and the correct program was selected (Fig.16C).

Fig 16. A. Master mix for CRE PCR, **B.** expected results in CRE PCR, **C.** CRE program **D.** 100bp ladder. **E.** results of CRE PCR and **F.** the repetition of CRE PCR.



Reagents	ul/sample
dNTPs	1
FW CRE 324	1
RV CRE 325	1
Taq Buffer	2
MgCl2	1.2
Taq	0.4
H2O	11.9
DNA	1.5
TOTAL VOLUME	20

Agarose gel

Afterwards, the PCR products had to be separated by electrophoresis via 2% agarose gel. As about the **agarose gel**, 200ml of 10x TBE (Tris/Borate/EDTA) was added in 1800ml water in order to make 1x running buffer. 250ml of it were put in a conical flask and 5g of agarose powder were added on it. This mix was warmed in the microwave till the powder to be diluted and the mix to be homogenous. Then, it was cooled under running water and 12μl of ethidium bromide was added on it. The mix was rinsed in a tray with well combs and was let for 40 minutes to coagulate. Afterwards, the gel was put in a gel box with 1x TBE and was ready for loading. 4μl of orange G (loading buffer) was added in the total volume of 20μl of each PCR product and 12μl of it was added in each well.

We had also to use positive and negative controls. C57BL/6 plays the role of WT mouse. Water plays the role of negative control. CRE is the positive control in CRE PCR (Fig.16.B).

A 100bp ladder was added in the first well that was an indicator of the bp of each different band. (Fig.16D)

The samples had to run at 120V for 45 minutes. DNA fragments are negatively charged, so they move towards the positive electrode. Afterwards, the gel was optimized via UV light in order to validate the genotyping of the mice (Based on the expected results Fig.16B).

CRE PCR was validated twice and the bands were as expected at 727bp. (Fig16.E, F)

Samples 1-6 were used as CRE (8 mice) whereas 7-14 as control (6mice).

Legislation

Of course, the mice were manipulated based on legislation and welfare recommended from the Federation of European Laboratory Animal Science Associations (FELASA). As a researcher, I was based on early indicators of intense agony, pain, suffering or impending death. These, are mentioned in the box below (Table 1).

Table 1: Humane endpoints in mice experiments

- **20% body weight loss**
- **15% body weight loss in 2 days**
- **Temperature drop >4°C**
- **Tumor size in mouse 1.2 X 1.2 cm**
- **Tumor ulceration**
- **Ascites > 10% B.W.**
- **Diarrhea > 48h**
- **Abnormal posture etc.**

Tamoxifen

Finally, the experiment included 8 mice (cre) **MHCII f/f Cdh5Cre** and 6 littermates (control) **MHCII f/f**.

2 months old mice were treated with Tamoxifen (Sigma, T5648-1G), in order to induce I-AB (MHCII) loss from TECs in endothelial cells, **(conditional knockout mice)** which was synthetized in the lab via the following protocol. Firstly, 20 tubes with 900μl of corn oil as well as 2,5 ml of 100% ethanol were placed in 37°C for 1h in the waterbath. 200mg of tamoxifen powder and 2ml of the 100% ethanol (mix) were also placed in the waterbath. The mix was vortexed till being homogenised and 100μl of it was placed in each tube of 900μl of corn oil. The tubes were vortexed and stayed in the water bath. When dissolved, they were stored at -20 ^{o}C.

The injections of tamoxifen lasted for 5 days in a row, in 2 months mice. 100μl of tamoxifen (10mg/ml) was injected intraperitoneally (IP) in each mouse.

Cell culture

LLC (Lewis lung carcinoma) cell line was used in order to induce cancer tumor in the lung.

Before surgeries, **LLC** cells had to be manipulated in the cell culture hood in sterility. The content of the vial was defrosted from -80°C and was put in 5ml of DMEM complete. Following, it was put in a medium flask in 10-15ml total volume. After 2 days the cells were split depending on their confluency, in order to be ready for the surgery. LLC cells are adherent cells. When split had to be done, the supernatant had to be discarded and 10ml of 1x PBS were added and discarded as well. Then Trypsin/EDTA was added for 1min in order for the cells to get off the bottom of the flask. Then Complete DMEM was added in order to stop the action of Trypsin/EDTA and then the correct dilution was done.

The medium used for their culture was **DMEM complete** that included: DMEM (Dulbecco's Modified Eagle Medium), Sodium Pyruvate, Sodium Bicarbonate, 1% P/S (Penicillin/Streptomycin), 1% L- Glutamine, 10% FBS (Fetal Bovine Serum) and NaOH (sodium hydroxide).

Mycoplasma

Furthermore, it was very important to do a trial for the Lewis Lung Cancer cell line in order to check for mycoplasma contamination before use. Mycoplasma PCR was used for that purpose.

C_{last} for mycoplasma PCR had to be 20-50μg/μl.

Mycoplasma PCR includes 2 ‘’serial’’ PCRs based on the table below (Table.2A) There was no product in the PCR, so no mycoplasma was found.

Table 2. Column 1st and 2nd:2 serial mycoplasma PCRs. Column 3rd and 4th:Program of the 2 serial PCRs

1st PCR	2nd PCR	Program for 1st PCR	Program for 2nd PCR
10µl DNA	2µl DNA from 1st PCR	94°C 30sec	94°C 30sec
2,5µl dNTPs	2,5µl dNTPs	94°C 30sec 55°C 2min (x30) 72°C 2min	94°C 30sec 55°C 2min (x30) 72°C 2min
2µl M89	2µl R2		
2µl R1	2µl R34		
2µl F1	2µl F2	72°C 2min	72°C 5min
2µl M79			
5µl 10X Taq buffer	5µl 10X Taq buffer		
0,2 µl Taq	0,2 µl Taq		
21,8µl H20	21,8µl H20		
2,5µl MgCl2	2,5µl MgCl2		
Vtotal=50µl	**Vtotal=50µl**		

Anaesthesia

2 week after the last injection of tamoxifen, Lewis Lung Cancer (LLC) cells had to be injected in the left lobe of the lung of each mouse in order to induce cancer after 2 weeks long. The whole procedure was done in anesthetized mice.

The anesthesia was made by the animal breeders and includes: ketamine, xylasine, atropine and water for injection (Table 3). The Ketamine/Xylazine combination is considered to be a very reliable anesthetic for mouse surgery.

Table 3. Mouse anesthesia cocktail (Animal facility,Fleming)

	Drug stock concentration[1] (mg/ml)	Volume used for cocktail	Volume of cocktail administered to mouse	**Dose administered to mouse**
Ketamine	100 mg/ml	2 ml	-	**100 mg/kg**
Xylazine	20 mg/ml	0.66 ml	-	**6.6 mg/kg**
Atropine	1 mg/ml	0.66 ml (1:10)		
W.F.I.	Sterile	6.66 ml	-	-
Combination Cocktail		10 ml total	0,05ml/10g	-

More specifically, the mice were anesthetized and a section was performed in order for the injection to be done in the left lobe of lung with the correct amount of LLC cells.

Regarding the dose of injected LLC cells (including 10% of matrigel), it was 150µl (2x 10^5 per mice) after standardization.

Matrigel is a soluble and sterile extract of basement membrane proteins derived from the EHS tumor that forms a 3D gel at 37 ◦C and supports cell morphogenesis, differentiation, and tumor growth. Matrigel, which primarily consists of laminin, collagen IV, and enactin, is used to mimic the ECM in cancer [93].

Then of course stitches were done in order for the wound to heal. In general mice have a very good healing ability. As already mentioned, then, we had to wait for 2 weeks in order for the lung cancer to be induced and potentially isolated.

Mouse Euthanasia

The cells were proliferated and the mice were sacrificed 15 days after the injection of LLC-OVA cells in the lung. The mice were sacrificed via the use of dry ice. Ethanol was used in order to decontaminate the belly and not hair to influence the surgery. A section was done in the belly from pelvis to chin. Then the main aorta was cut and blood perfusion was performed via the administration of PBS in the heart. Then the left lobe of lung was isolated and from it, the tumor only. The isolated lung tumors and a control lung were placed in PBS+ in different bizoo. PBS+ includes: 1X PBS, EDTA and FBS.

Isolated lungs

Following, the isolated tumor and healthy lung, had to be enzymatically digested. Each isolated tumor and the healthy lung were placed in 50ml falcons and were snipped with scissors. 2,5ml of collagenase mix were added at each of them. This mix includes 1mg/ml Collagenase type IV from *Clostridium histolyticum*, 1mg/ml DNase, 0,09mg/ml Dispase II, 10% FBS as well as HBSS.

They were placed for 30min in 37°C shaking incubator.

Afterwards, they were pushed through cell strainers (70μm), with the use of syringe plug, in order to filter the cancer cells. Then, PBS+ was added in each tumor, till 30ml. The mix was centrifuged at 300g for 6 minutes and the supernatant was discarded. The pellet was dissolved in 1ml of PBS+, PBS+ was added till 30ml and the whole mix was filtered once more.

Cell counting

Afterwards, the amount of cells was counted via the use of the Coulter machine. 10μl were taken from each sample and placed in 10ml of isoflow.

- ✓ Isoflow is an isotonic fluid specifically formulated for use in flow cytometers. It is carefully manufactured for low particle and fluorescence backgrounds to ensure superior signal to noise ratio measurements.

The coulter range was set at 6-24nm (above Tl) and the machine gave one number (coulter measurement) for each sample after calibration with 20ml isoflow. The amount of cells was measured: coulter measurement x 600000 [$2x10^4$x30(volume)] = a ($x10^6$)

We wanted for FACS: $\frac{5x10^6}{a} = \frac{50\mu l}{x}$ <=> we calculated x for each sample. (Table 3)

Then, each mix was centrifuged once more at 300g for 6minutes. The pellet this time was dissolved in PBS+ according to the amount of cells that it included. So, the volume of each sample had to be x μl after addition of PBS+ according to the table 4.

Table 4. Cell counting

		Coulter measurement	a ($x10^6$)	x (μl)
healthy		750	45	450
1	**control**	887	53,22	532.2
2	**control**	2375	142.5	1425
3	**control**	244	14.64	146.4
4	**control**	1315	78.9	789
5	**control**	1212	72.72	727.2
6	**control**	782	46.92	469.2
7	**Cre**	1191	71	714.6
8	**Cre**	1790	107.4	1074
9	**Cre**	895	53.7	537
10	**Cre**	2242	134	1345.2
11	**Cre**	549	32.94	329.4
12	**Cre**	5900	354	3540
13	**Cre**	4700	282	2820
14	**Cre**	4025	241.5	2415

The correct concentration of cells was finally used in order to be stained for **FACs** analysis. Before FACs, staining had to be standardized. The best antibodies had to be chosen and the correct amount of it in order to have the best experimental setup.

FACS

As already mentioned, the amount of cells of each tumor was stained with specific antibodies/markers depending on what we wanted to see each time. In Table 5 are mentioned some of the most important endothelial cell markers that were used during this project.

Table 5. Endothelial cell markers (modified from https://www.rndsystems.com/research-area/endothelial-cell-markers).

CD31/ PECAM-1	CD31, known as PECAM-1 (platelet/endothelial cell adhesion molecule 1), is a heavily glycosylated transmembrane homophilic adhesion protein that is highly expressed on endothelial cells and is required for migration of leukocytes, playing a key role in removing aged neutrophils from the body. The extracellular domain of CD31 is released during endothelial cell apoptosis. This fragment circulates in the serum of patients suffering from myocardial infarction, acute ischemic stroke,

	and multiple sclerosis [94].
CD80/B7-1 CD86/B7-2	Under certain conditions, ECs can serve as antigen presenting cells by expressing both MHC class I and class II molecules[97]. For example, hepatic sinusoidal ECs have the ability to express the costimulatory adhesion molecules CD80 (B7-1) and CD86 (B7-2), expression of which can be increased by ischemia/reperfusion of the rat liver.
Podoplanin	Podoplanin, also known as T1 alpha and Aggrus, is a mucin-type transmembrane glycoprotein with extensive O-glycosylation. It is expressed by lymphatic ECs, as well as by non-endothelial cells in some tissues. Podoplanin participates in regulation of lymphatic vascular formation and platelet aggregation.[97]
VE-cadherin	The cadherin superfamily consists of a large number of cell surface glycoproteins with cadherin repeats, upon which a Ca2+-dependent cell-cell adhesion depends. VE-cadherin is a major endothelial adhesion molecule controlling cellular junctions and blood vessel formation[98].

Following are the specific antibodies used in each different staining and the correct amount of each of them (Table 5). For each staining, a master mix was made according to table 6, including one fluorescent minus one (FMO), with no markers. The stainings were made in a 96well plate. Each well included 150µl sample and 250µl beads when it was a need.

Table 6. Amount of antibodies in each different staining

T regs staining	50µl
Zombie Al700	(1/30) 0.5
CD45 APC CY7	0,1
CD3 PE CY7	0,1
CD4 FITC	0,1
CD25 PE	0,2
CD16/32	0,5
INTRACELLULAR	
FOXP3 APC	0,3
PLUS BEADS	

T cytokines staining	50µl
Zombie Al700	(1/30) 0.5
CD45 PE CY5	0,1
CD8 APC CY7	0,1
CD16/32	0,5
INTRACELLULAR	
IFNg FITC	0,2
GRANZB PE	0,2

T enumeration staining	50µl
Zombie Al700	(1/30) 0.5
CD45 APC-Cy7	0,1
CD8a PERCP	0,1
CD4 FITC	0,1
DEXTRA PE	0,5
CD16/31	0,5
PLUS BEADS	

CD8 activation staining	50µl
Zombie Al700	(1/30) 0.5
CD8 APC CY7	0,1
PD1 PE CY7	0,2
TIGIT PE	0,2
CD44 FITC	0,1

CD4 activation staining	50µl
Zombie NIR (APC CY7)	(1/10) 0.5
CD4 Al700	0,1
PD1 PE CY7	0,2
TIGIT PE	0,2
CD44 FITC	0,1

Immune cell enumeration	50µl
Zombie Al700	(1/30) 0.5
CD45 APC-Cy7	0,1
F480 PE	0,1
LY6G APC	0,1
LY6C FITC	0,1

TIM3 APC	0,2
CD16/32	0,5

TIM3 APC	0,2
CD16/32	0,5

B220 PERCP	0,1
CD16/32	0,5
PLUS BEADS	

Tumor Burden	50µl
Zombie FITC	
INTRACELLULAR	
mcherry APC	0,2
PLUS BEADS	

MHCII in endo	50µl
Zombie Al700	(1/30) 0.5
CD45 Al700	0,1
CD31 PE	0,1
MHCII APC Cy7	0,05
podoplanin PE Cy7	0,1
CD16/32	0,5
INTRACELLULAR	
Vimentin 647 (-20,19474)	0,05

More specifically, in FACS analysis, single-cell suspensions were resuspended in FACS buffer (PBS, 2% FBS, 1.5 mM EDTA). Cells were stained at 4 °C for 30 min with fluorescent-conjugated antibodies or primary antibodies, followed by fluorescent-conjugated secondary antibodies. The antibodies' dilutions applied were according to manufacturers' instructions. For detection of intracellular antibodies, phosphorylated proteins or transcription factors, cells were fixed and permeabilized using the Fixation & Permeabilization Buffer Set (eBioscience) according to manufacturers' instructions. For calculation of absolute numbers of tumor cells (burden) or immune cells counting beads (123Count eBeads, Thermo Fischer Scientific) were used. Briefly, a known volume of counting beads was added to the same known volume of stained cells. The beads were counted along with cells. The absolute count of the cell population (A) was obtained by dividing the number of positive cell events (X) by the number of bead events (Y) and then multiplying by the bead concentration (N/V, where N = number of beads per test and V = test volume). $A = X/Y \times N/V$. FACS analysis was performed using FACSCANTO II (BD Biosciences) and data were analyzed using Flowjo (Tree star Inc) or DIVA software (BD Biosciences). All the results of FACS analysis are showed below at figures 17-24.

3. Results

We had some preliminary data that was the first indicator that endothelial cells play a role as atypical antigen presenting cells in lung cancer. Syngeneic immunocompetent mice were used. Lung orthotopic transplantation of LLC-OVA cells was done in 2 months old mice and the mice were sacrificed after 2 weeks. **FACS** analysis was done in the isolated tumor from the left lobe of lung. Firstly, it was shown that more than half of MHCII-expressing non-haemopoietic cells are expected to be TECs (CD31+). **TECs** constitute the majority of non-hematopoietic MHCII-expressing cells in the murine lung TME.

MHCII-positive lungs TECs express co-inhibitory but no co-stimulatory molecules. More specifically, it was shown that endothelial cells of lung LLC tumors express high levels of MHCII and the inhibitory molecule PDL-1 but low levels of costimulatory molecules (CD80, CD40, CD86). (Fig.17)

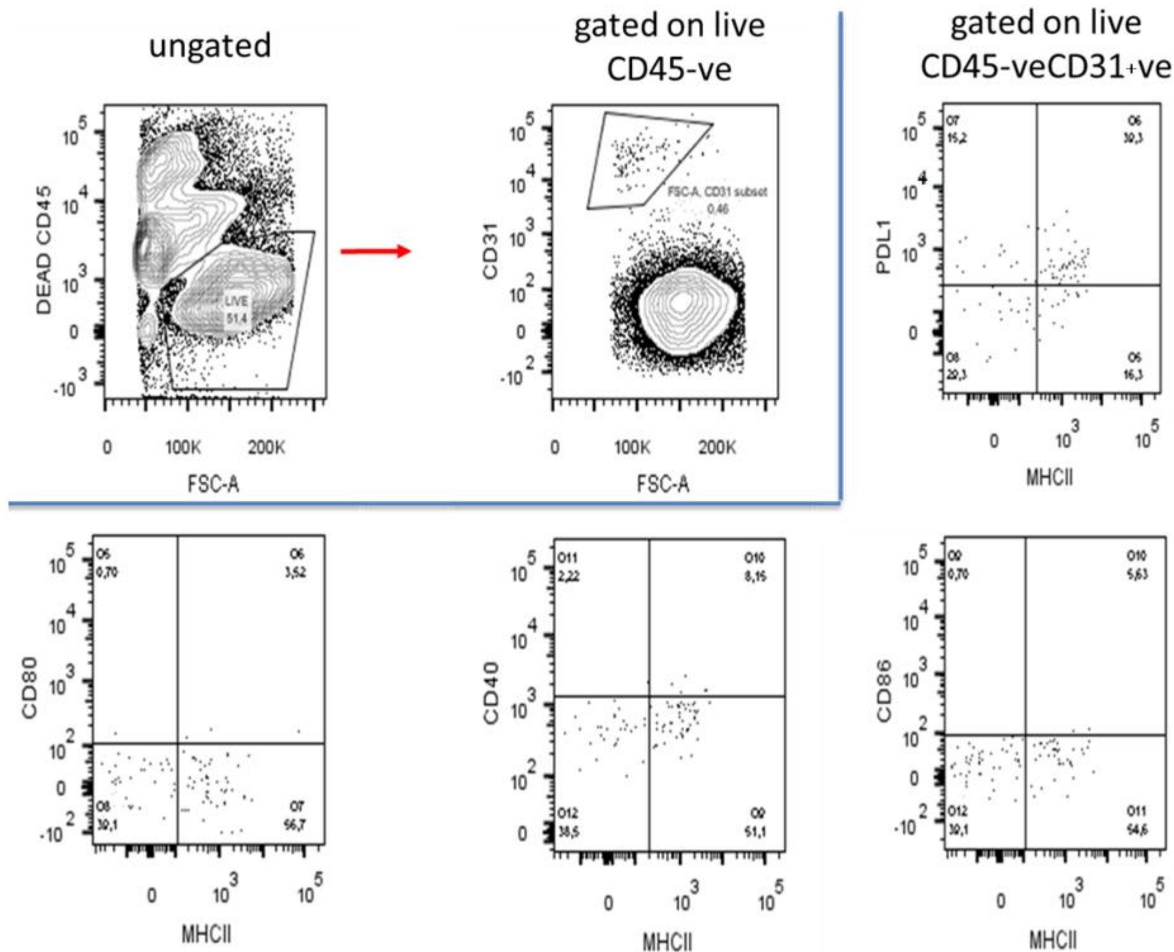


Fig.17 Inhibitory and Co-stimulatory molecules in endothelial cells of LLC tumors

As already mentioned above, the experiment included 8 experimental mice MHCII f/f Cdh5Cre and 6 control littermates MHCII f/f. When these mice were 2 months old, 100μl tamoxifen (10mg/ml) were injected to each of them that lasted for 5 days in a row. After 2 week, Lewis Lung Cancer (LLC) cells were injected in the left lobe of mice in order to induce cancer in the lung. The standardized dose was 150μl of LLC cells (2x 10^5 per mice), including 10% of matrigel. The mice were sacrificed 15 days after LLC-OVA injections and the tumor was analyzed via **FACS**. Above are the results.

It's shown in vivo that LLC cells grow faster in general in MHCII F/F Cdh5aCre mice (Fig. 18A and B) and the mice have statistical significant larger tumors comparing to control (MHCII F/F). Tumor Burden was measured via Zombie FITC that is a dead cell discriminator and mcherry APC (eBioscience, M11241) that is an intracellular stain.

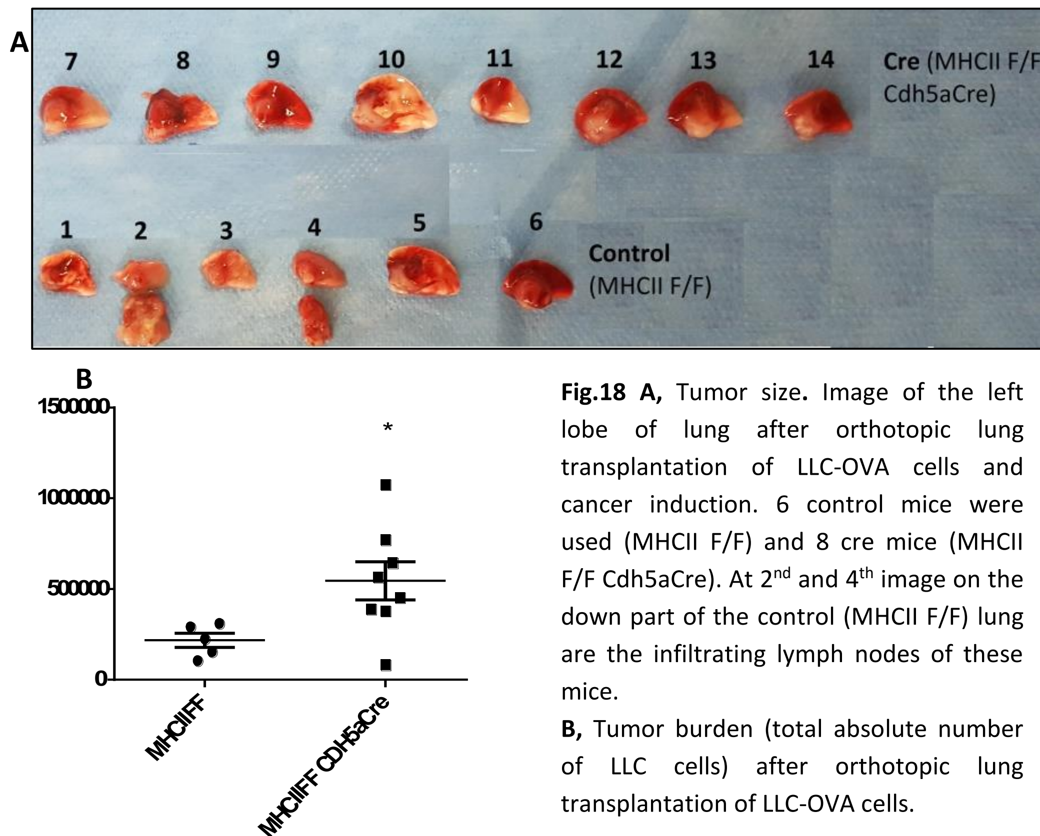


Fig.18 A, Tumor size**.** Image of the left lobe of lung after orthotopic lung transplantation of LLC-OVA cells and cancer induction. 6 control mice were used (MHCII F/F) and 8 cre mice (MHCII F/F Cdh5aCre). At 2nd and 4th image on the down part of the control (MHCII F/F) lung are the infiltrating lymph nodes of these mice.
B, Tumor burden (total absolute number of LLC cells) after orthotopic lung transplantation of LLC-OVA cells.

Expression of MHCII in CD31 PDPL NEG TM is statistically significant higher in homozygous loxP-flanked MHCII (MHCII F/F) than in the MHCIIF/F CDdh5aCre so the Cdh5aCre leaded to a deletion of MHCII allele in endothelial cells (19A). MFI of MHCII in endothelial cells was higher in the control group (Fig.19D)

MFI (Mean Fluorescence Intensity) refers to the fluorescence intensity of each event in average, represent the expression quantity of the parameter you chose on each event.

The staining used were: Zombie Al700, CD45 Al700 (Biolegend, 103128), CD31 PE (BD Pharmingen, 553373), MHCII APC Cy7(Biolegend, 107627), podoplanin PE Cy7(Biolegend,

127411), CD16/32 (Biolegend, 101310) and as about intracellular staining Vimentin 647(Abcam, ab194719).

Zombie is a dead cell discriminator. **CD45** is a general leukocyte marker. **CD31** is an endothelial cell marker. **Podoplanin** (PDPN) is expressed on many tumors and is involved in tumor metastasis. **CD16/CD32** is expressed on B cells, monocytes/macrophages, NK cells, granulocytes, mast cells, and dendritic cells. CD16 is low affinity IgG Fc receptor III (FcR III) and CD32 is FcR II. It was used for blocking.

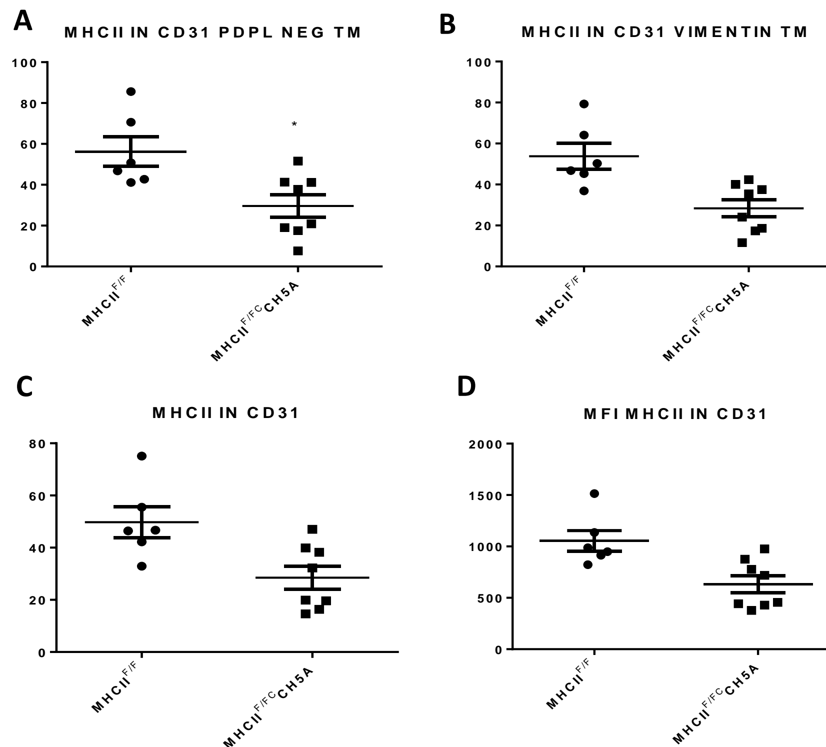


Fig.19 MHCII in endothelial cells (CD31)

CD4 and **CD8** absolute number (ABS.NO) is comparable in the two different groups of mice. We had the same result when using dextramers in CD45. (Fig.20)

MHC dextramer reagents are fluorescent labeled MHC multimers that can detect antigen specific T cells in fluid cell samples and solid tissues. More specifically LLC-OVA cells will initiate anti-tumor T cells responses (which can be detected with dextramers). MHC class II dextramers detect CD8 T cells with specificity and sensitivity.

The stainings used for T cell enumeration were: Zombie Al700, CD45 APC-Cy7(Biolegend, 103116), CD8a PERCP (BD Pharmigen, 553036), CD4 FITC (BD Pharmigen, 553651) , DEXTRA PE (DEXTRAMERS new NIH CD8 SIINFEKL, 35496) , CD16/31. **Beads** were also used for more accurate counting of cells.

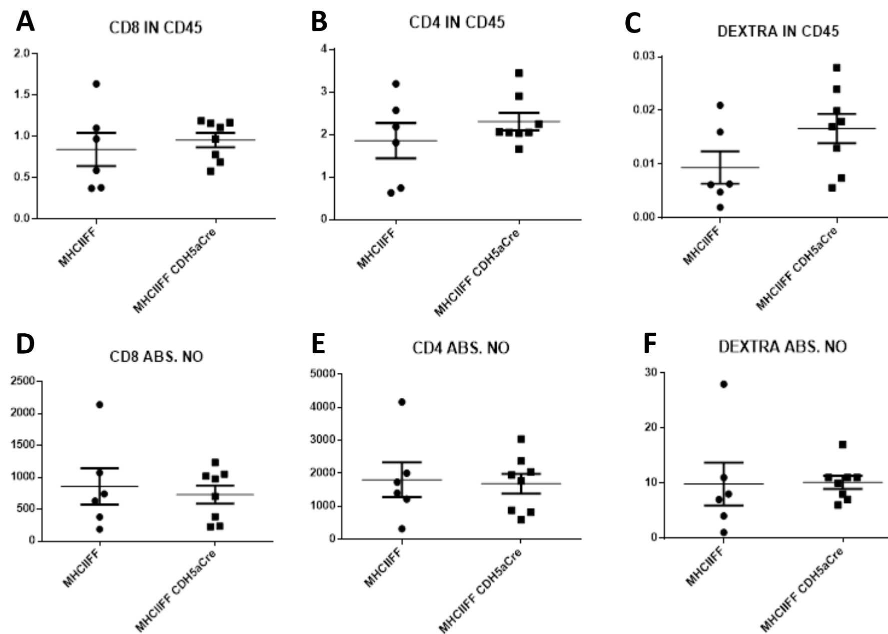


Fig.20 T cell enumeration in leukocytes (CD45) and their absolute number (ABS.NO).

Then we checked the CD4 and CD8 T cell activation (Fig.21, 22).

More specifically, we checked expression of major inhibitory molecules, (TIGIT, PD1, TIM-3) as well as an activation marker (CD44). (Fig.21)

Regarding the CD8 activation stain was used: Zombie Al700, CD8 APC CY7 (Biolegend, 100714), PD1 PE CY7 (Biolegend, 109110), TIGIT PE (Biolegend, 142103), CD44 FITC (BD Pharmigen, 553133), TIM3 APC (Biolegend, 119705), CD16/32 (Biolegend, 101310). **PD1, TIM3** and **TIGIT** act as T cell exhaustion Markers. **CD44** is a prominent activation marker which distinguishes memory and effector T cells from their naïve counterparts. It also plays a role in early T cell signaling events as it is bound to the lymphocyte-specific protein kinase and thereby enhances T cell receptor signaling.

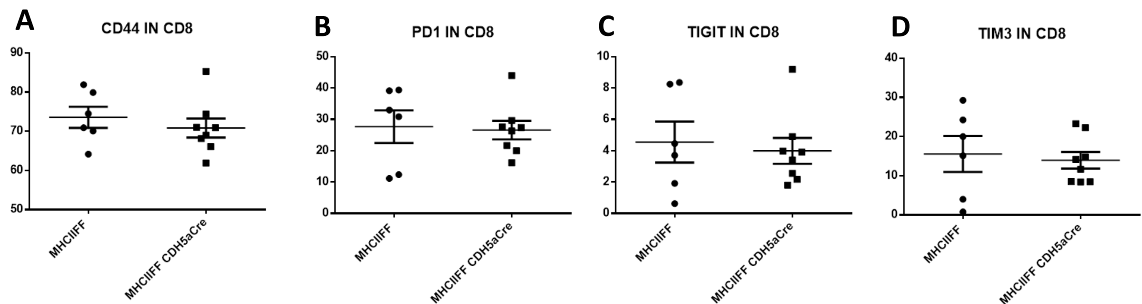


Fig.21 CD8 T cell activation. CD44 is a costimulatory molecule whereas PD1, TIGIT and TIM3 are co-inhibitory molecules.

As about T cell activation, CD44 had the same trend in MHCIIF/F in CD8 (Fig.21) and CD4 T cells (Fig.22). As about TIGIT and PD1, they were slightly lower in the surface of vascular endothelial. The only difference was in TIM3 that was higher in CRE mice in the surface of CD4 but the difference was not statistical significant.

In order to measure the CD4 T cell activation were used:

Zombie NIR (APC CY7), CD4 Al700 (eBioscience, 56-0042-82), PD1 PE CY7 (Biolegend, 109110), TIGIT PE(Biolegend, 142103), CD44 FITC(BD Pharmigen, 553133), TIM3 APC(Biolegend, 119705), CD16/32(Biolegend, 101310).

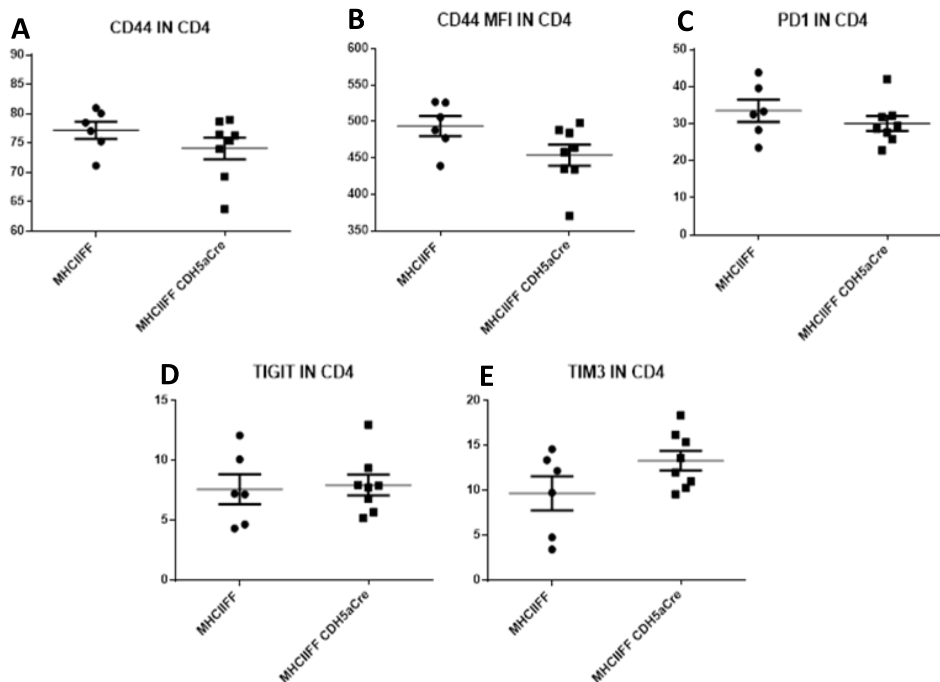


Fig.22 CD4 T cell activation. CD44 is a costimulatory molecule whereas PD1, TIGIT and TIM3 are co-inhibitory molecules.

In the scatter plots is shown that **Tregs** were higher in haemopoietic cells (CD45) as well as in CD4 T cells in the experimental versus the control group. (Fig.23) The absolute number of Tregs in the tumor of experimental mice was significantly higher showing the activation of the immune system in order to eliminate tumor that is bigger. (Fig.23)

Concerning Tregs staining were used as antibodies: Zombie Al700, CD45 APC CY7 (Biolegend, 103116), CD3 PE CY7 (eBioscience, 25-0031-82), CD4 FITC (BD Pharmigen, 553651), CD25 PE (BD Pharmigen, 553866), CD16/32 (Biolegend, 101310). As about intracellular staining for FOXP3, FOXP3 APC (Biolegend, 126407) was used as well as counting beads.

CD3 and **CD4** can be used as markers for T cells.

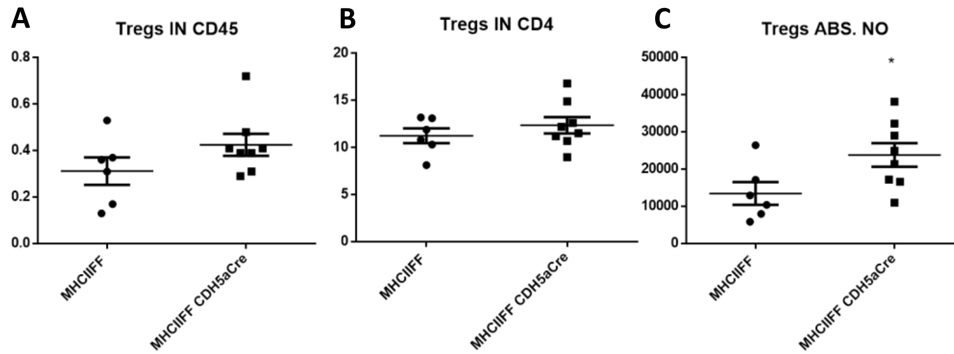


Fig. 23 Treg enumeration.

Then, the general **immune profile** was analyzed.

Macrophages and monocytes were almost the same in the 2 groups of mice. As about B cells, they were higher in the experimental group versus control but the difference was not statistical significant. They are antigen presenting cells and they are more active to eliminate the tumor. **Granulocytes** on the other hand were lower, but not statistical significant as well. (Fig. 24)

As about the immune profile and more specifically the immune cell enumeration, they were used: Zombie Al700, CD45 APC-Cy7 (Biolegend, 103116), F480 PE (eBioscience, 12-4801-82), LY6G APC (Biolegend, 127613), LY6C FITC (BD Pharmigen, 553104), B220 PERCP (eBioscience, 45-0452-82), CD16/32(Biolegend, 101310). Beads were also used.

More specifically, **F480** is as a major macrophage marker. **Ly6G** is a marker for monocytes, granulocytes and neutrophils. **Ly6C** is a monocyte-associated marker. **B220** also known as CD45R is expressed on B cells (at all developmental stages from pro-B cells through mature B cells), activated B cells, and subsets of T and NK cells.

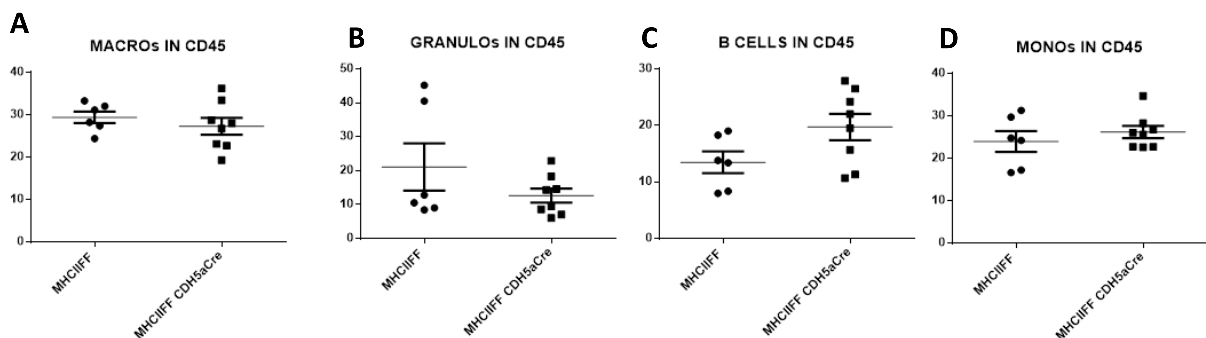


Fig.24 Immune profile

4. Discussion

Mouse models can recapitulate the complexity of human malignancy and immune contexture within the tumor microenvironment in order to predict efficiency and resistance to cancer therapy.

Endothelial cells (ECs) have been called "semiprofessional" antigen presenting cells (APCs) because they do not express certain costimulatory molecules yet they enhance T cell responses in vitro and are thought to stimulate allo responses in vivo [99–101].

Tumor endothelial cells that line tumor blood vessels are known to induce cancer metastasis and for that reason they are important targets in cancer therapy. There is a great need to focus on the influence of TECs in lung cancer.

The **preliminary data** was a first indicator **TECs** constitutes the majority of non-hematopoietic MHCII-expressing cells in the murine lung TME. Firstly, it was shown that more than half of MHCII-expressing non-haemopoietic cells are expected to be TECs (CD31+). This was the main reason to focus on this project. MHCII-positive lung TECs express co-inhibitory but no co-stimulatory molecules. More specifically, it is shown that endothelial cells of lung LLC tumors express high levels of MHCII and the inhibitory molecule PDL-1 but low levels of costimulatory molecules (CD80, CD40, CD86). (Fig.17) This is also proved by CM Card in his paper entitled: ''Emerging roles of lymphatic endothelium in regulating adaptive immunity''.[60] More specifically, it was mentioned that CD80 or CD86 co-stimulators are indispensable for the activation of naive lymphocytes and are absent from most endothelia.

The innovation on this paper was the use of **Cdh5aCRE** mouse in the concept of lung cancer in order to understand the role of endothelial cells as potential antigen presenting cells.

Alva JA et al. in the paper published in 2006 entitled VE-Cadherin-CRE-recombinase transgenic mouse: A tool for lineage analysis and gene deletion in endothelial cells highlights the useful role of cdh5acre mice in order to investigate the consequences of genetic manipulation within the vascular system.[102]

As already mentioned above, our **experiment** included 8 mice MHCII f/f Cdh5Cre and 6 littermates MHCII f/f. When the mice were 2 months old tamoxifen had to be given to them for 5 days in a row because CRE recombinase was tamoxifen inducible. After waiting for 2 weeks, LLC lung cells were injected in the left lobe of lung and after 2 more weeks, lung cancer was induced. The mice were sacrificed and the cells of the immune system were analyzed via FACS.

An indicator that the experiment worked correctly was the comparison of the size of the lung and by extension the existence of cancer. The mice that didn't have MHCII in the

surface of endothelial cells in the tumor of the lung had larger tumors and that was a proof that MHCII in endothelial cells plays an actual role in the controlling of the tumor (Fig.17A). This was validated by FACS analysis, and more specifically by the measurement of the tumor burden. In vivo LLC cells grow faster in MHCII F/F Cdh5aCre mice (Fig. 17B) that doesn't have MHCII in endothelial cells in the tumor.

Expression of MHCII in endothelial cells is statistically significant higher in homozygous loxP-flanked MHCII (MHCII F/F) than in the MHCIIF/F CDdh5aCre. It's an indicator that tamoxifen worked correctly, induced Cdh5aCre and leaded to a deletion of MHCII allele in the surface of endothelial cells. (19A)

In Figure 20, T cell enumeration was analyzed. Absolute number of **CD4** and **CD8** is comparable in the two different groups of mice. We had the same result when using dextramers. (Fig.20 D-F) So the deletion of MHCII in the surface of endothelial cells didn't affect CD4 or CD8 T cell number. Ideally we could expect CD4 and CD8 to be less in the mice with no MHCII in endothelial cells that had bigger tumors. For that reason we have to check the activation of them.

Then we checked the CD4 and CD8 T cell activation (Fig.21,22).

More specifically, we checked expression of major inhibitory molecules on their surface, (TIGIT, PD1, TIM3) as well as via the CD44. (Fig.21)

CD44 acts as an activation marker and it has the same trend on CD4 and CD8 T Cells.

There is a trend on the surface of CD4 and CD8 cells when MHCII is expressed on endothelial cells but still not statistical significant. This shows that CD4 and CD8 cells are more activated in this case and this explains the smaller tumor. We have to repeat this experiment in order to be sure for the existence of this trend.

MHCII was significantly higher in intratumoral endothelial cells in control mice with a significantly smaller tumor (Fig.18B). On the same mice CD44 (activation marker) is higher (Fig.19) and this shows that T cells are more activated in this case.

Also it's known that when CD44 is high, CD4 and CD8 cells are effector/memory cells and not naïve ones. Recognition of antigen in appropriate form, i.e. in association with costimulatory signals on the surface of professional antigen-presenting cells (APCs), leads to extensive T-cell proliferation and differentiation into effector cells. [103] When MHCII is expressed on the surface of endothelial cells, it activates T cells and they become effector T cells in that way. We propose that this is probably the case in our experimental results.

As about **TIGIT** and **TIM3** (Fig.22D,E) in the surface of **CD4 T cells**, they were slightly lower in **CD4** T cells when endothelial cells had MHCII on their surface maybe because endothelial cells have steal them from the surface of CD4 T cells while taking MHCII. This phenomenon is known as **Trogocytosis.** More specifically, we propose that endothelial cells take MHCII from the surface of CD4 T cells. It was shown in the preliminary data that endothelial cells express only co inhibitory molecules (Fig.17). TIGIT and TIM3 are co-

inhibitory molecules and their expression is different in **CD8** T cells' surface showing a possible explanation of the whole idea of atypical presentation via MHCII from endothelial cells in the context of lung cancer.

Tregs were higher in haemopoietic cells (CD45) as well as in $CD4^+$ T cells in the experimental versus the control group. (Fig.23) The absolute number of Tregs in the tumor of experimental mice was also significantly higher. (Fig.23) In that way, the mice that hadn't MHCII in the surface of endothelial cells, were able to stratify more Tregs showing a better activation of the immune system in order to eliminate the bigger tumor.

The last to be analyzed via FACs was the general **immune profile**. Macrophages and monocytes had the same trend comparing the 2 groups of mice. (Fig. 24) That's the case for **B** cells that are higher in this case being really important antigen presenting cells. There was also a trend in **granulocytes** but of course the whole experiment has to be repeated.

As about the **future experiments**, the first thing to be done is of course the **repetition** of this experiment in order to validate the results we have.

Furthermore, we can test our model in autochthonous **KRAS** mouse models. All mice strains to be used will be on Bl6 background and will express the I-AB MHCII gene. The KrasLSL-G12D mice will be crossed to I-AB-flox mice. Kras mice (B6.129S4-Krastm4Tyj/J, also known as KrasLSL-G12D), carries a Lox-Stop-Lox (LSL) sequence followed by the K-ras G12D point mutation. Cre recombination allows the expression of the mutant KRAS oncogenic protein. During this experiments, KrasLSL-G12D I-AB-flox mice will be crossed to the Cdh5(PAC)-CreERT2 that is already analyzed above. Lentiviruses (Lenti- LucOS) expressing cre-recombinase, luciferace and two MHCI and MHCII restricted ovalbumin (OVA) peptides will be instilled intratracheally to induce oncogenic KRAS activation and initiate tumorigenesis, as described [104]. OVA will initiate anti-tumor T cells responses (which can be detected with dextramers) and luciferace will enable us monitor tumor growth (DuPage et al., 2011). The time interval to lung adenocarcinoma development in KrasLSL-G12D is approximately 4 months. During the last weeks experimental and control (KrasLSL-G12D I-AB-flox) mice have to be treated with tamoxifen to induce I-AB (MHCII) loss from CAFs (conditional knockout mice).

Ex-vivo experiments are going to be the next step. **Cultures** will be set up in order to manipulate the endothelial and assess its effect.

A proposed future experiment would also be the functional characterization of MHCII-expressing TECs and TECs that don't express MHCII in primary human lung tumors. Of course, they could be analyzed by the use of **RNA sequencing**. In that way, transcription will be checked as well as pathways that are enriched in immune response.

Nanoparticles that target endothelial cells are already available and are ideal candidates to be used for this type of experiments. We can target locally the lung tumor with the use

of magnetic nanoflowers. This can be additive to the whole procedure followed during this project. More specifically, we can use the innovative **mNFLs** that carry antigens and adjuvants in order to target specifically endothelial cells in the tumor region and fight against cancer.

Taken all together, it's clear that a dysfunctional **endothelium** develops in response to the overexpression of pro-angiogenic factors in tumors. It is highly specialized and varies considerably from tissue to tissue and organ to organ.[1] Leakiness of tumor blood vessels is a major obstacle to therapeutic access and preclinical and clinical studies have shown that vascular normalization can augment even drug delivery to tumors. Integration of signals emanating from the TME and tumor cells trigger several immunosuppressive pathways in BECs that shape the outcome of antitumor immune responses.

This study suggests that TECs capture cancer-associated antigens and present MHCII-restricted cancer peptides in order to inhibit adaptive immune resistance. There is a possibility that trogocytosis occur as well that is already mentioned above.

Trogocytosis is the transfer of plasma membrane fragments from one cell to another without cell death induction. This process is mediated by receptor signaling following cell–cell contact.[105] Several recent studies reported that Trogocytosis of MHC class I (MHCI) and, more importantly in our case, of MHC class II (MHCII) occurs not only between T cells and APCs, but between a wide variety of cell types including APCs–APCs, APCs–NK cells, tumor cells–T or NK cells, etc. suggesting that the type of cell receiving such MHC may impact antigen-specific T cell activation.[105] As we showed that endothelial cells act in lung cancer as atypical antigen presenting cells and we also propose that Trogocytosis maybe occur.

We imagine a future scenario where reprograming TECs can shut down their tolerogenic functions in lung cancer will restore anti-tumor T cell responses and induce tumor immune rejection with minimal concerns for serious side effects. The whole procedure that is used to target endothelial cells ideally has to be used for therapeutic purpose in the near future. Monotherapy will be great provided if it will be accurate and effective. Combinational therapy will also be a solution in order to relief lung cancer patients mainly via combination of the herein proposed therapy with immunotherapy. We have to manipulate the antigen presentation machinery of endothelium in order to have an impact in the patient.

References

1. Dudley, A. C. Tumor Endothelial Cells. *cold spring Harb. Perspect. Med.* (2012).
2. Https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/key-statistics.html. American cancer society. https://www.cancer.org/cancer/non-small-cell-lung- (2019).
3. Huston, D. P. The Biology of the Immune System. *Jama Netw.* **278**, 1804–01814 (2015).
4. Chaplin, D. D. Overview of the Immune Response. *J Allergy Clin Immunol.* **125**, (2010).
5. MD, V., MH, K., RD, S. & MJ, S. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* 235–71 (2011).
6. Bonilla, F. A. & Oettgen, H. C. Adaptive immunity. *J. Allergy Clin. Immunol.* **125**, S33–S40 (2010).
7. Stuart E. Turvey, and D. H. B. Innate Immunity. *J Allergy Clin Immunol.* **125**, 1–17 (2011).
8. Y, L. & G, Z. Cancer and innate immune system interactions: translational potentials for cancer immunotherapy. *J Immunother* **35**, 299–308 (2012).
9. Du, Y., Chen, X., Huang, Z.-M., * X.-H. Y. & Niu2, Q. Increased Frequency of Foxp3+ Regulatory T Cells in Mice with Hepatocellular Carcinoma. *Asian Pacific J. Cancer Prev.* **13**, 3815–3819 (2012).
10. Steinman, R. M. Dendritic cells: Understanding immunogenicity. *Eur. J. Immunol.* **37**, 53–60 (2007).
11. McDevitt, H. O. & Sela, M. Genetic control of the antibody response. I. Demonstration of determinant-specific differences in response to synthetic polypeptide antigens in two strains of inbred mice. *J. Exp. Med.* **122**, 517–531 (1965).
12. Mcdevitt, B. Y. H. O. *et al.* GENETIC CONTROL OF THE IMMUNE RESPONSE MAPPING OF THE IR-1 Locus. *J. Exp. Med.* **135**, (1972).
13. Kambayashi, T. & Laufer, T. M. Atypical MHC class II-expressing antigen-presenting cells: Can anything replace a dendritic cell? *Nat. Rev. Immunol.* **14**, 719–730 (2014).
14. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunology. *Nat. Rev.* **392**, 245–252 (1998).
15. RN, G. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell* 287–99 (1994).
16. RH., S. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* 1065–8 (1992).
17. Chen, D. S. & Mellman, I. Oncology meets immunology: The cancer-immunity cycle. *Immunity* **39**, 1–10 (2013).
18. Rizvi, N. A. *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science (80-.).* **348**, 124–128 (2015).
19. Tran, E. *et al.* Cancer Immunotherapy Based on Patient with Epithelial Cancer.

Science (80-.). **9**, 641–646 (2014).
20. Remark, R. *et al.* The non-small cell lung cancer immune contexture: A major determinant of tumor characteristics and patient outcome. *Am. J. Respir. Crit. Care Med.* **191**, 377–390 (2015).
21. Lakins, M. A., Ghorani, E., Munir, H., Martins, C. P. & Shields, J. D. Cancer-associated fibroblasts induce antigen-specific deletion of CD8 + T Cells to protect tumour cells. *Nat. Commun.* **9**, 1–9 (2018).
22. Chen, D. S. & Mellman, I. Elements of cancer immunity and the cancer-immune set point. *Nature* **541**, 321–330 (2017).
23. Campana, S., De Pasquale, C., Carrega, P., Ferlazzo, G. & Bonaccorsi, I. Cross-dressing: an alternative mechanism for antigen presentation. *Immunol. Lett.* **168**, 349–354 (2015).
24. Hida, K., Maishi, N., Annan, D. A. & Hida, Y. Contribution of tumor endothelial cells in cancer progression. *Int. J. Mol. Sci.* **19**, 1–12 (2018).
25. Maia, J., Caja, S., Strano Moraes, M. C., Couto, N. & Costa-Silva, B. Exosome-Based Cell-Cell Communication in the Tumor Microenvironment. *Front. Cell Dev. Biol.* **6**, 1–19 (2018).
26. Conway, E., Collen, D. & Carmeliet, P. Molecular mechanisms of blood vessel formation. *Trends Biochem. Sci.* **22**, 251–256 (2001).
27. Carmeliet, P. Angiogenesis in life, disease and medicine. *Nature* **438**, 932–936 (2005).
28. Dorland, W. A. N. *''Endothelium" at Dorland Medical Dictionary*. (1980).
29. Jason M. Butler, Hideki Kobayashi, and S. R. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nar Rev Cancer* **10**, 138–146 (2010).
30. Carmeliet, P. & Jain, R. K. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat. Rev.* **10**, (2011).
31. Schaaf, M. B., Garg, A. D. & Agostinis, P. Defining the role of the tumor vasculature in antitumor immunity and immunotherapy. *Cell Death Dis.* (2018). doi:10.1038/s41419-017-0061-0
32. DM McDonald & Baluk, P. Significance of blood vessel leakiness in cancer. *Cancer reasearch* 5381–5 (2002).
33. Castermans, K. & Griffioen, A. W. Tumor blood vessels, a difficult hurdle for infiltrating leukocytes. *Biochim. Biophys. Acta - Rev. Cancer* **1776**, 160–174 (2007).
34. Turley, S. J., Cremasco, V. & Astarita, J. L. Immunological hallmarks of stromal cells in the tumour microenvironment. *Nat. Rev. Immunol.* (2015). doi:10.1038/nri3902
35. Carmeliot, P. INHIBITION OF THE GLYCOLYTIC ACTIVATOR PFKFB3 IN ENDOTHELIAL CELLS INDUCES TUMOR VESSEL NORMALIZATION, IMPAIRS METASTASIS AND IMPROVES CHEMOTHERAPY. *Cancer Cell.* **30**, 968–985 (2017).
36. Frederique Vegran, Romain Boidot, Carine Michiels, P. S. and O. F. Lactate Influx through the Endothelial Cell Monocarboxylate Transporter MCT1 Supports an NF- k B / IL-8 Pathway that Drives Tumor Angiogenesis. *Mol. Cell. Pathobiol.* **71**, 2550–2561 (2011).

37. Ruan, G. & Kazlauskas, A. Lactate Engages Receptor Tyrosine Kinases Axl , Tie2 , and Vascular Endothelial Growth Factor Receptor 2 to Activate Phosphoinositide 3-Kinase / Akt and Promote Angiogenesis *. *J. Biol. Chem.* **288**, 21161–21172 (2013).
38. Joseph W. Franses, Aaron B. Baker, V. C. C. and E. R. E. Stromal Endothelial Cells Directly Influence Cancer Progression. *Sci Transl Med.* **3**, (2011).
39. Bandyopadhyay, S. *et al.* Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. *Nat. Med.* **12**, 933–938 (2006).
40. Hida, K., Akiyama, K., Ohga, N., Maishi, N. & Hida, Y. Tumour endothelial cells acquire drug resistance in a tumour microenvironment. *J. Biochem.* **153**, 243–249 (2013).
41. Hida, K., Hida, Y. & Shindoh, M. Understanding tumor endothelial cell abnormalities to develop ideal anti-angiogenic therapies. *Cancer Sci.* **29**, 459–466 (2008).
42. Carman, C. V, Martinelli, R., Pober, J. S. & Carman, C. V. T Lymphocyte-endothelial interactions : emerging Understanding of Trafficking and Antigen-Specific immunity. *Front. Immunol.* **6**, (2015).
43. AL, R. *et al.* Endothelial cells present antigens in vivo. *BMC Immunol.* (2004).
44. AS, D., SV, F., JW, F., A, T. & PJ, M. The detailed distribution of HLA-A, B, C antigens in normal human organs. *Transplantation* 287–92 (1984).
45. Hughes, C. C., Savage, C. O. & Pober, J. S. Endothelial cells augment T cell interleukin 2 production by a contact-dependent mechanism involving CD2/LFA-3 interaction. *J. Exp. Med.* **171**, 1453–67 (1990).
46. Shiao, S. L., McNiff, J. M. & Pober, J. S. Memory T Cells and Their Costimulators in Human Allograft Injury. *J. Immunol.* **175**, 4886–4896 (2014).
47. Shiao, S. L. *et al.* Human Effector Memory CD4+ T Cells Directly Recognize Allogeneic Endothelial Cells In Vitro and In Vivo. *J. Immunol.* **179**, 4397–4404 (2014).
48. JS, P., T, C., Jr, G. M., P, L. & CS, R. Inducible expression of class II major histocompatibility complex antigens and the immunogenicity of vascular endothelium. *Transplantation* **41**, 141–6 (1987).
49. Bella, S. Della & Detmar, M. Tumor-Associated Lymphatic Vessels Upregulate PDL1 to Inhibit T-Cell Activation. *Front. Immunol.* **8**, 1–13 (2017).
50. Lukacs-kornek, V., Fletcher, A. L., Hedegaard, D. L., Fletcher, A. L. & Lukacs-kornek, V. The Role of Lymphatic Endothelial Cells in Liver Injury and Tumor Development. *Front. Immunol.* **7**, 1–7 (2016).
51. SJ., T. Regulated release of nitric oxide by nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. *Nat Immunol.* **12**, 1096–1104 (2012).
52. Capotosti, F., Lund, A. W., Corthe, P., Swartz, M. A. & Winter, C. H. Steady-State Antigen Scavenging, Cross-Presentation, and CD8 + T Cell Priming: A New Role for Lymphatic Endothelial Cells. *J. Immunol.* (2014). doi:10.4049/jimmunol.1302492
53. Tewalt, E. F., Cohen, J. N., Rouhani, S. J., Engelhard, V. H. & Mueller, C. G. Lymphatic endothelial cells – key players in regulation of tolerance and immunity. *Front. Immunol.* **3**, 1–6 (2012).

54. Lund, A. W. *et al.* VEGF-C Promotes Immune Tolerance in B16 Melanomas and Cross-Presentation of Tumor Antigen by Lymph Node Lymphatics. *Cell Rep.* **1**, 191–199 (2012).
55. Fletcher, A. L. *et al.* Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. *J. Exp. Med.* **207**, 689–697 (2010).
56. Cohen, J. N. *et al.* Lymph node–resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J. Exp. Med.* **207**, 681–688 (2010).
57. Podgrabinska, S. *et al.* Inflamed lymphatic endothelium suppresses dendritic cell maturation and function via Mac–/ICAM-1-dependent mechanism. *J. Immunol.* **183**, 1767–1779 (2015).
58. A, S. *et al.* Dynamic regulation of CD8 T cell tolerance induction by liver sinusoidal endothelial cells. *J Immunol.* 4107–14 (2010).
59. EF, T. *et al.* Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood* 120:4772–82 (2012).
60. CM, C., SS, Y. & MA., S. Emerging roles of lymphatic endothelium in regulating adaptive immunity. *J Clin Invest* **124**, 943–52 (2014).
61. D, K. *et al.* Cutting edge: MHC class II expression by pulmonary nonhematopoietic cells plays a critical role in controlling local inflammatory responses. *J Immunol* **185**, 3809–13 (2010).
62. A, L. *et al.* Efficient presentation of exogenous antigen by liber endothelial cells to CD8+ T cells results in antigen-speciic T-cell tolerance. *Nat Med.* 1348–54 (2000).
63. A, K., T, H., A, I. & T, U. Vascular endothelial cells provide T cells with costimulatory signals via the OX40/ gp34 system. *J Leukoc Biol* 111–118 (2000).
64. S, K. *et al.* ICOS-ligand, expressed on human endothelial cells, costimulates Th1 and Th2 cytokine secretion by memory CD4+ T cells. *Natl Acad Sci USA* 6198–6203 (2002).
65. Karmann K, Min W, Fanslow WC, P. J. Activation and homologous desensitization of human endothelial cells by CD40 ligand, tumor necrosis factor, and interleukin 1. *J Exp Med* 173–182 (1996).
66. Savinov, A. Y., Wong, F. S., Stonebraker, A. C. & Chervonsky, A. V. Presentation of antigen by endothelial cells and chemoattraction are required for homing of insulin-specific CD8+ T cells. *J. Exp. Med.* **197**, 643–56 (2003).
67. Valujskikh, A., Lantz, O., Celli, S., Matzinger, P. & Heeger, P. S. Cross-primed CD8+T cells mediate graft rejection via a distinct effector pathway. *Nat. Immunol.* **3**, 844–851 (2002).
68. De Sanctis, F., Ugel, S., Facciponte, J. & Facciabene, A. The dark side of tumor-associated endothelial cells. *Semin. Immunol.* **35**, 35–47 (2018).
69. Zang, X. *et al.* Tumor associated endothelial expression of B7-H3 predicts survival in ovarian carcinomas. *Mod pathol.* **23**, 1104–1112 (2010).
70. Huang, X. *et al.* Lymphoma endothelium preferentially expresses Tim-3 and

facilitates the progression of lymphoma by mediating immune evasion. *J. Exp. Med.* **207**, 505–520 (2010).

71. Urban, J. Genes that Distinguish Physiological and Pathological Angiogenesis. *Cancer Cell.* **5**, 379–390 (2010).
72. Mulligan, J. K., Day, T. A., Gillespie, M. B., Rosenzweig, S. A. & Young, M. R. I. Secretion of vascular endothelial growth factor by oral squamous cell carcinoma cells skews endothelial cells to suppress T-cell functions. *Hum. Immunol.* **70**, 375–382 (2009).
73. Young, J. K. M. and M. R. I. Tumors induce the formation of suppressor endothelial cells in vivo. *Cancer Immunol Immunither.* **5**, 379–390 (2010).
74. Casós, K. *et al.* Tumor cells induce COX-2 and mPGES-1 expression in microvascular endothelial cells mainly by means of IL-1 receptor activation. *Microvasc. Res.* **81**, 261–268 (2011).
75. Munn, D. H. & Mellor, A. L. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. *J. Clin. Invest.* **117**, 1147–1154 (2007).
76. Gregory T. Motz, Stephen P. Santoro, Li-Ping Wang, Tom Garrabrant, Ricardo R. Lastra, Ian S. Hagemann, Priti Lal, Michael D. Feldman, Fabian Benencia, and G. C. Tumor Endothelium FasL Establishes a Selective Immune Barrier Promoting Tolerance in Tumors. *Nat Med.* **20**, 607–615 (2014).
77. Lund, A. W. *et al.* VEGF-C Promotes Immune Tolerance in B16 Melanomas and Cross-Presentation of Tumor Antigen by Lymph Node Lymphatics. *Cell Rep.* **1**, 191–199 (2012).
78. Hendry, S. A. *et al.* The role of the tumor vasculature in the host immune response: Implications for therapeutic strategies targeting the tumor microenvironment. *Front. Immunol.* **7**, 1–21 (2016).
79. Monvoisin, A. *et al.* VE-cadherin-CreER T2 Transgenic Mouse : A Model for Inducible Recombination in the Endothelium. *Dev. Dyn.* 3413–3422 (2006). doi:10.1002/dvdy.20982
80. Branda, C. S., Dymecki, S. M. & Cre-, S. Talking about a Revolution: The Impact of Site-Specific Recombinases on Genetic Analyses in Mice. *Dev Cell.* **6**, 7–28 (2004).
81. Feil, R. Conditional somatic mutagenesis in the mouse using site-specific recombinases. *Handb. Exp. Pharmacol.* **178**, 3–28 (2007).
82. Feil, R. *et al.* Ligand-activated site-specific recombination in mice. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 10887–90 (1996).
83. Feil, R., Metzger, D. & Chambon, P. Regulation of Cre Recombinase Activity by Mutated Estrogen Receptor Ligand-Binding Domains. *Biochem. Biophys. Res. Commun.* **757**, 752–757 (1997).
84. Metzger, D., Clifford, J., Chiba, H. & Chambon, P. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 6991–5 (1995).
85. Zhang, Y. *et al.* Inducible site-directed recombination in mouse embryonic stem cells. *Oxford Univ. Press* **24**, 543–548 (1996).
86. CAMBA, R. & DIAZANI MOR, M. A. Temporally-controlled site-specific mutagenesis

in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ERT and Cre-ERT2 recombinases. *Nucleic Acids Res.* **45**, 229–241 (1962).
87. Kühbandner, S. *et al.* Temporally controlled somatic mutagenesis in smooth muscle. *Genesis* **28**, 15–22 (2000).
88. Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70–71 (2006).
89. Feil, S., Valtcheva, N. & Feil, R. Inducible Cre Mice. *Methods Mol. Biol.* **530**, 343–363 (2009).
90. Proença, J. T., Coleman, H. M., Connor, V., Winton, D. J. & Efstathiou, S. A historical analysis of herpes simplex virus promoter activation in vivo reveals distinct populations of latently infected neurones. *J. Gen. Virol.* **89**, 2965–2974 (2008).
91. Wang, Y. *et al.* Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature* **465**, 483–486 (2010).
92. Hashimoto, K., Joshi, S. K. & Koni, P. A. A conditional null allele of the major histocompatibility IA-beta chain gene. *Genesis* **32**, 152–153 (2002).
93. Hughes, C. S., Postovit, L. M. & Lajoie, G. A. Matrigel : A complex protein mixture required for optimal growth of cell culture. *Proteomics* 1886–1890 (2010). doi:10.1002/pmic.200900758
94. Riccieri, V., Stefanantoni, K. & M. Vasile et al. Abnormal plasma levels of different angiogenic molecules are associated with different clinical manifestations in patients with systemic sclerosis. *Clin. Exp. Rheumatol.* **29**, 46–52 (2011).
95. Sakimoto, S., Marchetti, V. & E. Aguilar et al., “. CD44 expression in endothelial colony-forming cells regulates neurovascular trophic effect. *JCI* **2**, (2017).
96. Mund, J. A., Estes, M. L., Yoder, M. C., Ingram, D. A. & and J. Case, “. Flow cytometric identification and functional characterization of immature and mature circulating endothelial cells. *rteriosclerosis, Thromb. Vasc. Biol.* **32**, 1045–1053 (2012).
97. J. Mai, A., Virtue, Shen, J., Wang, H. & Yang, and X. F. An evolving new paradigm: endothelial cells–conditional innate immune cells. *J. Hematol. Oncol.* **6**, (2013).
98. Vestweber, D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscler. Thromb. Vasc. Biol.* **28**, 223–232 (2008).
99. M, V., H, Y., J, de V. & M, K. Antigen presentation by human dermal microvascular endothelial cells. Immunoregulatory effect of IFN-γ and IL-10. *J Immunol* 5734–5741 (1994).
100. H, K., CD, S. & J, S. Stimulation of mature unprimed $CD8^+$ T cells by semiprofessional antigen- presenting cells in vivo. *J Exp Med* 1291–1302 (1992).
101. FM, M.-B. *et al.* Activated murine endothelial cells have reduced immunogenicity for $CD8^+$ T cells: a mechanism of immunoregulation? *J Immunol* 4182–4189 (2000).
102. Alva, J. A. *et al.* VE-cadherin-cre-recombinase transgenic mouse: A tool for lineage analysis and gene deletion in endothelial cells. *Dev. Dyn.* **235**, 759–767 (2006).
103. Berard, M., Tough, D. F. & Edward, T. Qualitative differences between naive and

memory T cells. *Immunology* 127–138 (2002).

104. Manley, G. *et al.* Endogenous T cell responses to antigens expressed in lung adenocarcinomas delay malignant tumor progression. *Cancer Cell.* **32**, 736–740 (2017).
105. Nakayama, M. Antigen presentation by MHC-dressed cells. *Front. Immunol.* **5**, 1–8 (2014).