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THE ROLE OF MARCH1 IN THE B16 MELANOMA MODEL

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This memoir :
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RÉSUMÉ

Les mécanismes de l'immunosurveillance des tumeurs commencent à être compris et sont appuyés par un multitude de données expérimentales et cliniques. Il est évident que la coopération entre tous les bras du système immunitaire est requise pour une réponse anti-tumorale optimale. Cependant, le rôle inhabituel des cellules T CD4+ dans l'éradication des tumeurs est maintenant bien démontré. Ce concept était initialement problématique, car la plupart des tumeurs non-hématopoïétiques n'expriment pas de CMH de classe II, qui est nécessaire pour la reconnaissance par les cellules T CD4+. Il est probable que cette réponse dépende des cellules présentatrices d'antigènes qui infiltrent la tumeur et présentent aux cellules T CD4+ des antigènes tumoraux via leur CMH de classe II. Ici, nous cherchons à étudier le rôle de MARCH1, une E3 ubiquitine ligase qui cible le CMH de classe II et le relocalise vers les endosomes de recyclage, dans le contexte du modèle expérimental de mélanomes B16. Ainsi, nous comparerons la souris sauvage et la souris MARCH1 KO dans ce modèle. Nous proposons que l'inoculation des tumeurs B16 chez la souris MARCH1 KO génèrera de plus grosses tumeurs que chez la souris sauvage, car l'expression de surface stabilisée du CMH de classe II génère des dysfonctions intrinsèques chez les cellules dendritiques. Nos résultats démontrent que la souris MARCH1 KO développe des tumeurs plus agressives et plus larges que la souris sauvage, associé avec une infiltration réduite de cellules T CD4+ dans les tumeurs. Ceci semble être associé à une activation déficiente des cellules T CD4+ dans les ganglions lymphatiques par les cellules dendritiques.

Mots clés: CMH de classe II, présentation antigénique, MARCH1, cancer.

ABSTRACT

The mechanisms of cancer immunosurveillance are beginning to be understood and the concept is being supported by tremendous experimental and clinical data. While all arms of the immune system are required for maximal anti-tumor responses, the importance of CD4+ T cells in tumor eradication is now well established. However, this concept was initially problematic, as most non-hematopoietic tumors do not express MHC class II molecules at their surface, which are necessary for recognition by CD4+ T cells. It is most likely that the early response is dependent on infiltrating APCs that uptake tumor antigens and present them to CD4+ T cells via their MHC class II molecules. We thus sought to study the impact of MARCH1, an E3 ubiquitin ligase which interacts with MHC class II molecules and limits their cell surface exposure, in the context of the B16 melanoma model. Comparing wild-type mice and MARCH1-deficient mice, we posited that MARCH1-deficient mice would react poorly to the B16 tumor challenge as stabilized MHC class II expression leads to severe impairments in DCs. Our findings demonstrate that MARCH1 KO mice developped more aggressive and larger tumors than their wild-type littermates. This was seemingly due to an defective DC-mediated priming of CD4+ T cells in the lymph nodes, resulting in a significantly reduced CD4+ TIL population.

Key words: MHC class II, antigen presentation, MARCH1, cancer.

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ABBREVIATION LIST

APC – Antigen presenting cell

CD – Cluster of differentiation

cDC – Conventional dendritic cell

CIITA – Class II-transactivator

CTL – Cytotoxic T cell

DC – Dendritic cell

dDC – Dermal dendritic cell

DMBA - 7,12-Dimethylbenz[a]anthracene

DNA – Deoxyribonucleic acid

FDA – Food and Drug Administration

IFN – Interferon

IL – Interleukin

LPS – Lipopolysaccharide

MAPK - Mitogen-activated protein kinases

MHC – Major Histocompatibility

PAMP – Pathogen-associated molecular pattern

pDC – Plasmacytoid dendric cell

PIC – Pre-integration complex

TCR – T cell receptor

TDLN – Tumor-draining lymph node

TEC – Thymic epithelial cell

TIDC – Tumor-infiltrating dendric cell

TIL – Tumor-infiltrating lymphocyte

TLR – Toll-like receptor

TNF – Tumor necrosis factor

Treg – Regulatory T cell

UV – Ultra-violet

CHAPTER 1: LITERATURE REVIEW

The role of MARCH1 in the development of B16 melanoma.

In this memoir, we studied the role of MARCH1 in the development of experimental B16 melanoma in mice. MARCH1 is an E3 ubiquitin ligase that is mainly expressed in antigen-presenting cells. In dendritic cells, MARCH1 ubiquitinates the cytoplasmic tail of the MHC class II molecule, promotes its internalization and regulates T cell activation *in vitro*. The CD4+ T cell-mediated tumor eradication in the context of MHC class II-negative tumors is supported by massive experimental evidence. Therefore, in this chapter, we reviewed MHC class II-mediated antigen presentation and the B16 melanoma model.

1.0 Major Histocompatibility Complex molecules and antigen presentation

Unlike the B cell arm of the adaptive immune response, a protective T cell response relies on the partial intracellular proteolysis of antigens from pathogenic and malignant cells and the binding of the resulting peptides onto the Major Histocompatibility Complex (MHC) molecules. All vertebrates possess the MHC, which is a large cluster of conserved genes, including the three types of peptide-binding glycoproteins (MHC molecules) and other essential genes implicated with the function of these molecules. Here, I will review only the two classical types of MHC molecules, the MHC class I and class II, which play a key role in the regulation of adaptive immune responses to endogenous and exogenous protein antigens.

The genes implicated in the MHC class I and class II antigen presentation are subject to important allelic variation, mostly at the sequence level where the interaction with the peptide occurs. In most vertebrates, there are several genes for MHC class I and II, which probably arose from gene duplication (1). In mice, depending on the strain, there are three genes for MHC class I (H2-D, H2-K and H2-L) and two genes for MHC class II (I-A and I-E). In humans, there are three genes for MHC class I (HLA-A, HLA-B and HLA-C) and three genes for MHC class II (HLA-DR, HLA-DQ and HLA-DP). The two MHC molecules are very similar in their three-dimensional structure and both bind peptides to engage the T cell receptor (TCR), but differ by their subunit composition. Furthermore, the

conformation of the peptide:MHC complex is different (2).

There are two proteolytic systems that generate these peptides. Peptides derived from the cytosol, for example in a cell infected by an intra-cellular pathogen or in a malignant cell, are degraded by a barrel-shaped complex called the proteasome. The proteasome recognizes ubiquitin-tagged molecules that have been targeted for degradation. Several proteolytic components of the proteasome are also induced by Interferon-γ and they differ by their cleavage specificities (3, 4). The proteasome with Interferon-γ-induced components is called the immunoproteasome.

It is the MHC class I molecules that bind cytosol-derived peptides, which are degraded by the proteasome and immunoproteasome and are expressed by all nucleated cells (5). Cytotoxic CD8+ T cells then recognize their cognate peptide bound to a MHC class I molecule. Cytotoxic T lymphocytes (CTLs) therefore recognize and kill infected or malignant cells. On the other hand, cell exogenous proteins, transmembrane proteins and extracellular pathogens, that are therefore not accessible to the proteasome, are internalized by phagocytosis or endocytosis and enter a vesicular pathway for processing. These peptides are then loaded onto the MHC class II molecule, whose expression is mainly restricted to antigen-presenting cells, and the complex migrates toward the cell surface to be presented to naive CD4+ T cells (6). Thus, dendritic cells (DCs) present MHC class II complexed peptides to naïve CD4+ T cells, which causes their differentiation into different types of effector and memory T cells. T-cell mediated immunity is thus highly dependent on MHC class II molecules. Also, although there is an apparent division of labor between CD4+ and CD8+ T cells, there is an important crosstalk between the two subtypes (Figure 1.1).

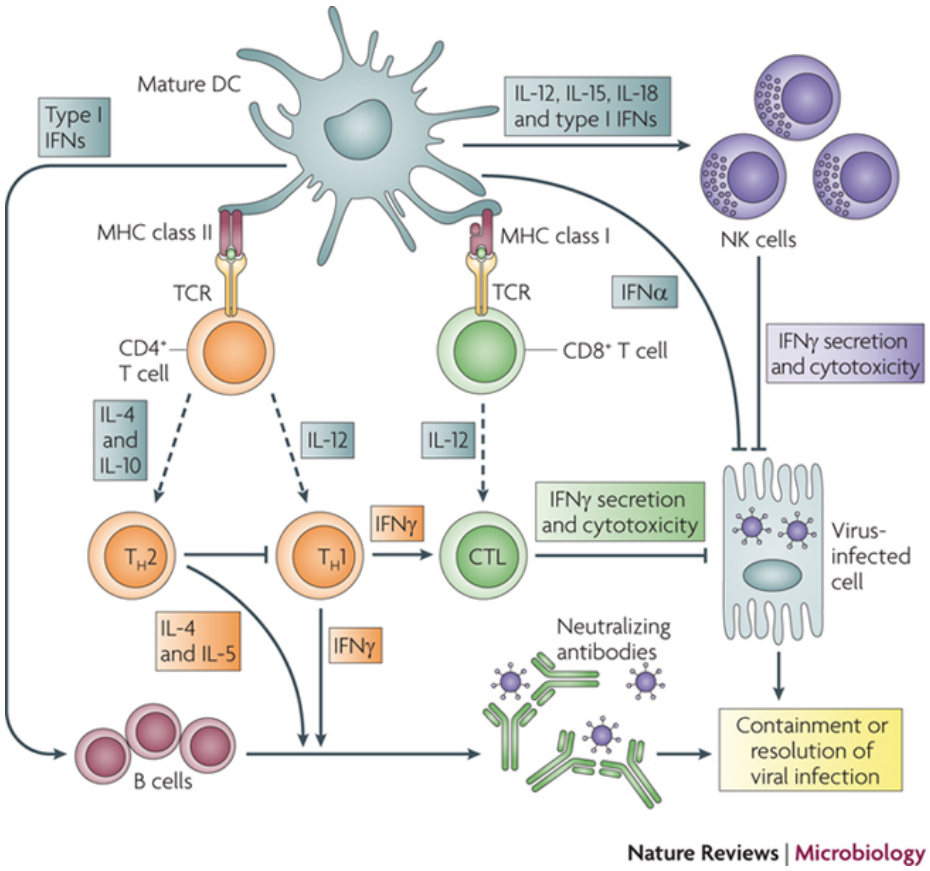

Figure 1.1: Illustration of the cross-talk between CD4+ and CD8+ T cells during the containment of a viral infection.

The uptake of an antigen, in this case, a viral antigen, leads to the maturation of the DC to the proximal lymph node, where it can interact and prime both naive CD4+ and CD8+ T cells. The CD4+ and CD8+ TCR engage with the MHC class II and class I respectively. In addition, the activated DC secretes various cytokines that promote T cell survival and differentiation, but can also activate other cell type such as natural killer (NK) cells. Depending on the signal, naive CD4+ T cell may differentiate into a particular subtype (here, only T_{H1} and T_{H2} are depicted). Both T_{H1} and T_{H2} help the antibody response. The T_{H1} cells can further stimulate the activation and expansion of CD8+ T cells by the secretion of IFN-g. Also, beyond helping CD8+ T cells in the early activation phase, it has been demonstrated that CD4+ T cells are required at the time of priming for the development of long-lasting memory CD8+ T cells (7-9). The interaction between CD4+ and CD8+ T cells is central to tumor immunity and will be described later. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Lambotin, M., et al. (2010). "A look behind closed doors: interaction of persistent viruses with dendritic cells." Nat Rev Microbiol 8(5): 350-360.), copyright (2016).

1.0.1 Regulation of MHC class II expression

MHC class II molecules are essential for the antigen-specific immune responses by T lymphocytes and are central to the maintenance of self-tolerance. Given this important role, their expression is tightly regulated at the transcriptional and post-translational levels.

First, the MHC II locus, which includes the structural genes and the promoter, is highly conserved in vertebrates. The transcription of this locus depends on several DNA-binding transcription factors, as well as the master regulator of MHC class II, the MHC II trans-activator (CIITA), which does not bind DNA (10). CIITA constitutive expression is limited to all MHC class II+ cells, most notably antigen-presenting cells (APCs), such as dendritic cells, B cells and macrophages (11). However, it is important to note that MHC class II expression may be induced in non-hematopoietic cell types under inflammatory conditions to amplify the T cell response at inflammation sites. Furthermore, CIITA expression can be induced or repressed by cytokines such as IFN-**γ**, LPS, IL-4, IFN-**β**, IL-10, nitric oxide and TGF-**β**, and depends upon the maturation state of the cell (11-13). Additionally, both CIITA-dependent and independent epigenetic alterations can regulate MHC class II expression (14).

In addition to *de novo* synthesis of MHC molecules, the surface expression of peptide:MHC II complexes may be modulated post-translationally by ubiquitination, adding an extra level of regulation. It has been demonstrated that a single conserved lysine residue in the cytoplasmic tail of the **β** subunit of MHC class II can be targeted by the ubiquitin machinery (15, 16). Ubiquitination is a post-translational modification critical for many cellular processes, such as protein degradation and intracellular transport (17, 18). In APCs, ubiquitination of the cytoplasmic tail of the loaded MHC class II molecule promotes its efficient endocytosis and trafficking, thereby negatively regulating CD4 T cell-mediated immunity (15, 16).

1.0.2 Ubiquitination

Ubiquitination is a protein modification pathway for cytosolic, membrane-bound and nuclear proteins. Although commonly associated with proteolysis, reversible ubiquitination of proteins also leads to non-proteolytic fates, such as cellular trafficking (18). Ubiquitination consists of a three-step cascade mechanism (Figure 1.2): the initial activation of the ubiquitin group, its conjugation and then ligation to the protein substrate. The first step is catalyzed by an E1 ubiquitin-activating enzyme and consists of ATP-dependent generation of an ubiquitin-adenylate intermediate, that is loaded onto an E2 ubiquitin-conjugating enzyme. Next, the E3 protein ligase carries simultaneously the loaded E2 enzyme and the substrate protein to promote the formation of a thioester bond between the ubiquitin and specific residues of the substrate protein (19). Ubiquitin moieties can be added to different residues of a protein, generally to a lysine, although serine, methionine, cysteine and threonine can act as ubiquitin acceptors too. Furthermore, as the ubiquitin molecule itself possesses seven lysine residues, the molecule can be itself ubiquitinated. Thus, a ubiquitin molecule may be repetitively ubiquitinated (polyubiquination). Alternatively, one (monoubiquitination) or many (multi-monoubiquitination) sites of a substrate protein may be ligated by a single ubiquitin molecule (20). This highlights the flexibility and diversity of the ubiquitin system. Ubiquitinated proteins are then recognized by ubiquin-binding proteins or receptors, for example at the proteolytic domain of the proteasome complex (21).

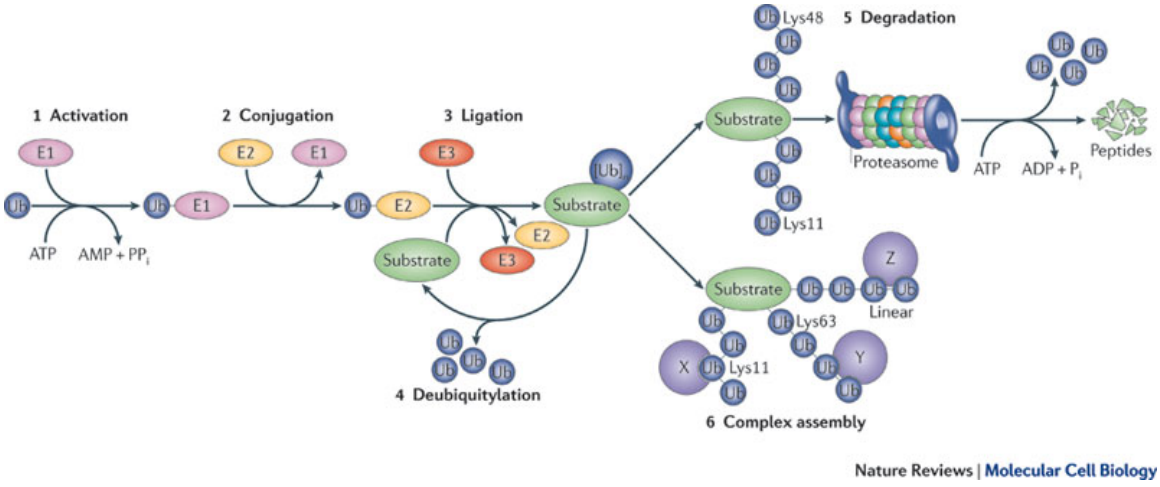

Figure 1.2: A schematic view of the ubiquitin machinery.

This figure illustrates the three-step cascade mechanism of the ubiquitin machinery. The small protein ubiquitin is loaded by an E1 ubiquitin-activating enzyme in an ATP-dependent reaction onto an E2 ubiquitin-conjugating enzyme. Then, an E3 protein ligase act as an adaptor and binds both the loaded E2 enzyme and the substrate protein to facilitate the peptide bond between ubiquitin and specific residues of the substrate. As different linkages of ubiquitin are possible, ubiquitinated proteins are recognized by linkage-specific ubiquitin-binding domains. As depicted, ubiquitinated proteins may be targeted to degradation by the proteasome, or can induce interaction with other proteins. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Vucic, D., et al. (2011). "Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death." Nat Rev Mol Cell Biol 12(7): 439-452.), copyright (2016).

1.0.3 MARCH1

MARCH1 is part of the Membrane-Associated Ring-CH (MARCH) family members, numbered to 11, that were first identified as mammalian homologs of Kaposi's sarcoma associated herpesvirus proteins (22, 23). The viral proteins target and ubiquitinate MHC class I, leading to its rapid endocytosis and degradation, thereby promoting immune evasion and viral persistence (24-26). Similarly to their viral homologs, most of the 11 members target membrane-bound immune-associated proteins, but only two members of this family, MARCH1 and MARCH8, were found to act specifically on MHC class II molecules. Both transmembrane proteins, they share characteristics that are not found in other MARCH family members. They share an identical transmembrane domain (26), which is the domain associated with substrate recognition, and as expected, they share similar targets: both can also target the immune co-stimulatory molecule CD86, the transferrin receptor and Fas (CD95). However, their tissue expression pattern differs: while MARCH1 expression is mainly limited, but not restricted to secondary lymphoid organs such as lymph nodes and spleen, MARCH8 expression seems more ubiquitous, with highest peaks in lungs and pancreas (26).

Their apparent redundancy was disproved in knock-out mice. The MARCH1-deficient mouse demonstrates a quasi-absence of ubiquitinated MHC II, and its isolated B cells and DCs express constitutively high levels of MHC class II (27). On the opposite, the

MARCH8-deficient mouse showed similar levels of MHC class II ubiquitination compared to its wild-type counterpart and had normal expression levels of MHC class II on isolated B cells and DCs. Thus, this suggests that MARCH1 is a physiological E3 ubiquitin ligase of APCs *in vivo* in mice. In humans, shRNA studies confirmed the same hypothesis (28).

The stabilization of MHC class II surface expression by the MARCH1 deficiency leads to several abnormalities that have been described in the MARCH1 KO mouse. Indeed, it has been demonstrated that this contributes to several DC anomalies such as reduced expression of co-stimulatory molecules CD4 and CD8, and impaired production of IL-12 and TNF-α upon stimulation (29). Furthermore, the MARCH1 deficiency results in significantly reduced thymic-derived regulatory T cells (Tregs) population (30). In the thymus, the MHC class II expression promotes the development of CD4+ T cells, including natural Tregs (31, 32). This is in part mediated by MARCH1, as ubiquitinated MHC class II in thymic DCs promotes the development of natural Tregs by an unclear mechanism that does not implicate avidity control (30).

Furthermore, this August, two groups highlighted an apparent division of labor between MARCH1 and MARCH8 in the regulation of MHC class II surface expression in the thymus for the development of CD4+ T cells (33, 34). As already mentioned, MARCH1 regulates the cell surface expression of MHC class II in thymic APCs to support the development of natural Tregs. Similarly, MARCH8 rather regulates the expression of MHC class II in thymic epithelial cells (TECs) to promote the selection of CD4+ T cells. Additionally, the groups found that the transmembrane domain of CD83, already found to bind the transmembrane domain of MARCH1 to prevent its interaction with its target (35), inhibits MARCH8 and encourages stabilized MHC class II surface expression in TECs. Thus, the coordinated regulation of MHC class II surface expression on TECs by MARCH8 and CD83 has a crucial role on CD4+ T cell selection. These new findings propose new roles for MARCH proteins in the development of CD4+ T cells.

In peripheral tissues, the steady-state expression of MARCH1 in immature DCs, whose function is to capture and process antigens, limits the surface expression of pro-

inflammatory molecules such as MHC class II. Therefore, MHC class II molecules are retained intra-cellularly by oligo-ubiquitination of their cytoplasmic tail. However, upon pro-inflammatory stimuli, such as signaling via patterns-associated molecular patterns (PAMPs), which down-regulate MARCH1 expression and protein stability, the maturation of DCs, whose function is rather to initiate T cell immunity, is associated with an increased surface expression of MHC class II (Figure 1.3). Accordingly, the down-regulation of MARCH1 upon maturation signals permits the rapid and efficient trafficking of MHC class II molecules to the cellular membrane, in order to support antigen presentation by APCs (15, 30).

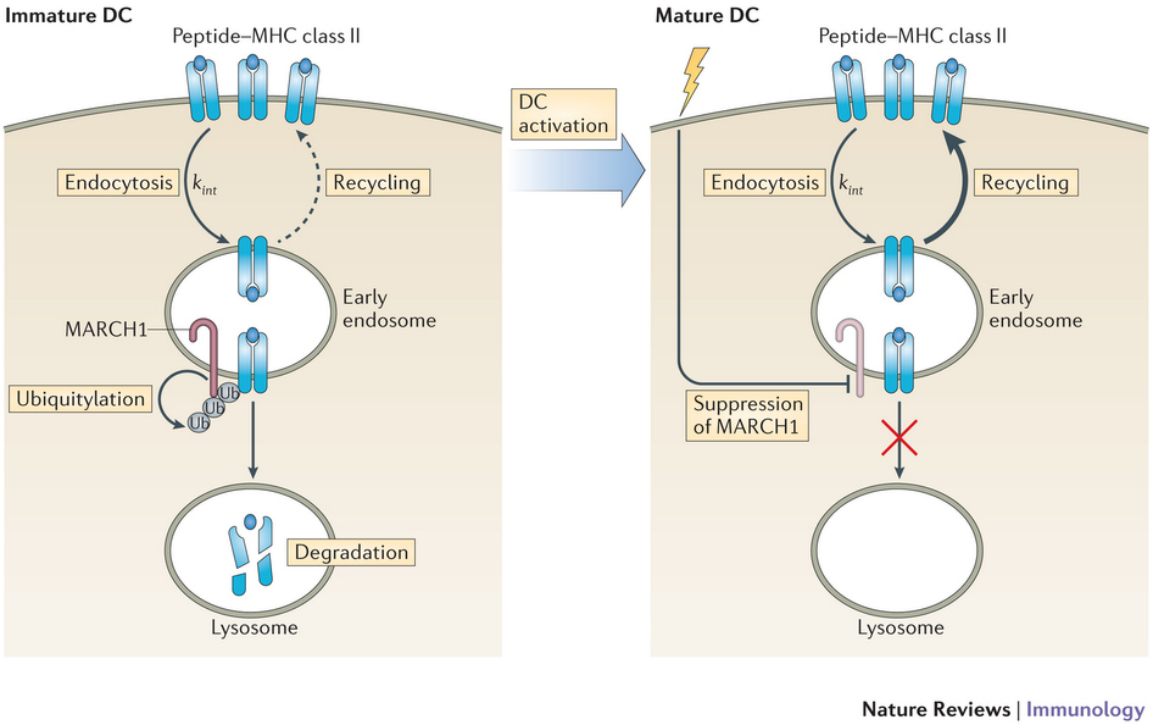

Figure 1.3: Schematic representation of the function of MARCH1 in maturing DCs.

In immature DCs, MARCH1 is expressed and ubiquitinates the cytoplasmic tail of the MHC class II molecule, which promotes its endocytosis. Consequently, immature DCs express low levels of MHC class II on their surface, as MHC class II molecules are retained in the cell's endosomes. However, upon DC activation by a maturation stimulus, MARCH1 expression is suppressed and thus, MHC class II molecules are no longer ubiquitinated. Therefore, MHC class II molecules are less efficiently endocytosed, leading to a high surface expression of MHC class II molecules. Reprinted by permission from Macmillan

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As the MHC class II molecule is critical for efficient cell-mediated immune responses, we can suppose that MHC class II expression influences greatly the anti-tumor response (36). This may suggest a role for MARCH1 in the anti-tumor immunity, but also as a potential target for immunotherapy.

1.0.4 Regulation of MARCH1 expression in APCs

As the mounting of an effective immune response against a pathogen or a malignant cells demands generous resources, the process of antigen presentation must be done under the appropriate conditions. Furthermore, as MARCH1 is an important regulator of antigen presentation, its expression must be tightly regulated.

As already highlighted, maturation signals such as TLR signalling by PAMPs will induce the up-regulation of surface expression of MHC class II and co-stimulatory molecules like CD86 on APCs, in order to stimulate a CD4-mediated response. This is orchestrated by the diminution of MARCH1 expression. It has been demonstrated that LPS treatment of DCs, which signals through the TLR4, leads to a drop in MARCH1 mRNA levels. Furthermore, MARCH1 proteins are inherently unstable and have a short half-life. Therefore, upon a maturation stimulus, the levels of MARCH1 protein are rapidly decreased (37).

The only cytokine that has been found to date to up-regulate the expression of MARCH1 is Interleukin-10 (IL-10). This cytokine was originally described by Mosmann and Fiorentino in the late 1980's (38). They elaborated that cytokines were acting as cross-regulators to maintain the clonal dichotomy between T_{H1} and T_{H2} cells. It was known at the time that interferon-gamma (IFN-$\boldsymbol{\gamma}$) was produced, among other cytokines, by T_{H1} cells as an autocrine growth factor but also as a suppressor of proliferation and activity of T_{H2} cells. The group discovered and described IL-10 as a T_{H2}-specific cytokine that inhibited IFN-$\boldsymbol{\gamma}$

synthesis in T_{H1} cells (39).

Later, other groups found that IL-10 treatment of monocytes increased intracellular concentrations of MHC class II, leading to a diminution of MHC class II on the cell surface (40). It was then found that this effect was mediated by MARCH1 (41). Thus, IL-10 was considered as an immunosuppressive cytokine that limited the activation of CD4+ T cells by down-regulating antigen presentation in APCs and to protect the host against immunopathology that may occur upon excessive immune activation (42).

Nowadays, a more complete and nuanced picture of IL-10 is accepted: IL-10 is a potent immunoregulatory cytokine. Its production is temporally and spatially restricted, it is produced later in the immune response, after the secretion of pro-inflammatory cytokines such as TNF-$\boldsymbol{\alpha}$ and IFN-$\boldsymbol{\gamma}$. Also, it is secreted by subsets of CD4+ T cells and other hematopoietic cells such as monocytes, macrophages, B lymphocytes, but also non-hematopoietic cells such as keratinocytes (43). As mentioned, this pleiotropic cytokine inhibits antigen-specific T_{H1}-mediated response by preventing their proliferation and the production of IFN-$\boldsymbol{\gamma}$, but also IL-2, IL-4, IL-5 and TNF-$\boldsymbol{\alpha}$. Furthermore, to downplay the immune response, IL-10 reduces the antigen-presenting capacities of monocytes and macrophages by limiting surface expression of MHC class II molecules and co-stimulatory molecules and suppresses their effector functions, notably the secretion of pro-inflammatory cytokines and chemokines (44, 45). Moreover, IL-10 acts on DCs to restrict cytokine secretion, mostly IL-12, and trafficking to lymph nodes (46). On the other hand, IL-10 has also various immunostimulatory functions: it acts as growth and differentiation factor for B lymphocytes, thymocytes and mast cells (47-49).

Finally, the implication of IL-10 in cancer remains controversial and suggest a context-dependent effect (50). Because IL-10 has the potential to generate tolerogenic responses, this cytokine was often associated with cancer persistence (51). However, other findings surprisingly propose that in certain cancer models, IL-10 may be associated with the induction of a T cell-mediated anti-tumor immunity (52, 53). Therefore, as MARCH1 is an effector of the IL-10-mediated response, this prompted us to study the impact of

MARCH1 in cancer.

1.1 Skin cancer and melanoma

While the rate of cancer incidence has been stable or slowly increasing over the past 30 years, both in men and women from all ages, skin cancer is one of the fastest rising cancer in Canada. It is expected that a 72% increase of new cases of melanoma will occur between 2007 and 2032 (54). Melanoma is the malignancy of the pigment-producing cells of the skin, the melanocytes. It is the most severe form of skin cancer and is one of the most common type in adolescents and young adults (54, 55). Although the prognosis is relatively good when detected early, it decreases rapidly with increased thickness of the lesion. However, melanoma remains often misdiagnosed and is characterized by its highly aggressive nature to invade and metastasize (56, 57). Exposure to ultraviolet (UV) radiation through exposure to sunlight and tanning beds appears to be a major risk factor for melanoma (58). As stratospheric ozone depletion is an ongoing process and increases the proportion of UV rays reaching the Earth's surface, there are considerable concerns for human health, including chronic sun damage of the skin and the eyes with increased incidence and severity. Although a great deal of sensitization campaigns against artificial UV exposure were initiated, human-induced climate changes are inevitable and pose a major challenge on human health (59).

Recent treatments include targeted therapies and immunotherapy, as conventional radiotherapy and chemotherapy are inadequate against melanoma (60). Targeted therapies, for example BRAF inhibitors (vemurafenib), are used in melanoma cases that harbour a *BRAF* mutation, which account for approximately 50% of melanoma cases (61). The *BRAF* gene encodes a kinase implicated in the mitogen-activated protein kinase (MAPK) pathway that mediates cellular responses to growth signals (62). Mutations in this gene most often result in an elevated kinase activity, leading to pathogenesis (61). However, BRAF inhibitors are not associated with durable anti-tumor responses, as melanoma cells rapidly acquire resistance through re-activation or over-activation of cellular pathways (63, 64). The most promising therapy currently is immunotherapy. The United States Food and Drug Administration (FDA) recently approved two drugs: ipilimumab (monoclonal antibody

anti-CTLA-4) and pembrolizumab (monoclonal antibody anti-PD1) are both immune checkpoint inhibitors that block negative regulators of the T cell response (65, 66). Both demonstrated some clinical successes, but these treatments showed a durable response only in a small subset of patients and cases of relapses and immune-mediated side effects were not uncommon (60, 67). Taken all together, these facts explain why melanoma is a considerable public health burden and efforts are needed to find new potential therapeutic targets.

1.1.1 The biology of melanocytes

The skin is the body's largest organ. It is highly complex: it is multilayered and expresses regional variation throughout the body. It is composed of three layers: the epidermis, the dermis and the hypodermis. The most superficial layer, the epidermis, consists of a continuously renewing stratified tissue, which demonstrates progressive differentiation (68). It is composed of different cell types, all from an embryonic origin: keratinocytes, melanocytes, Langerhans cells and Merkel cells. The basal layer of the epidermis, the stratum germinativum or stratum basale, is composed of mitotically active keratinocytes which migrate superficially and confer structural integrity, and immigrant cells such as melanocytes, Langerhans cells and Merkel cells (69).

Melanocytes are the melanin-producing cells: they promote melanogenesis and transfer the melanin-containing organelle, the melanosome, to neighbouring keratinocytes (70). The melanosome is the lysosome-related organelle that confer pigment and photo-protection to tissues (71). The survival of melanocytes at the basal layer is supported by growth factors, such as basic fibroblast growth factor, endothelin-1, melanocyte stimulating hormone and stem cell factor, produced by basal layer keratinocytes (69). The cell body of melanocytes is often extended below the basal layer, to the dermis. However, the dendrites may reach keratinocytes up to more superficial stratum of the epidermis. A single melanocyte can reach up to 36 keratinocytes, a formation called an epidermal-melanin unit (72). The melanosome is relocated from the perinuclear cytoplasm of the melanocyte, toward its dendrite tip via actin- and microtubules-based motor proteins (73). Then, the melanosome is transferred into keratinocytes, where it may be found individually or membrane-bound

into complexes that are degraded by lysosomal enzymes as the keratinocyte differentiates and moves toward the most superficial stratum of the epidermis (69, 74). The melanin released from the melanosome then aggregates near the nucleus of the keratinocytes and assembles into « supranuclear melanin caps », as to protect the nucleus from UV-induced damage (75, 76).

UV light generates DNA photo-damage and apoptosis of many cell types of the skin (77), but can also suppress local immune responses by acting on Langerhans cells. UV radiation on the skin has been shown to reduce the population of Langerhans cells in the skin, not via apoptosis but rather by inducing their migration toward sub-cutaneous lymph nodes (78). Furthermore, studies demonstrated that UV exposure altered the morphology of these cells (79) and impaired their antigen-presenting capacities (80). Melanocytes induce the production of melanin upon UV light stimulation, which in keratinocytes, filters and attenuates the radiation by scattering, absorption and dissipation by heat, accompanied by redox reactions and electron transfer processes (81). Thus, melanin acts as broadband UV absorbent by its radical scavenging activities (82).

Despite these protection mechanisms, melanocytes may acquire genetic alterations, either due to UV rays or spontaneously. These genetic aberrations confer survival and growth advantage over surrounding cells. These cells thrive at the expense of others and go for a first round of clonal expansion. The initial mutations, generally in oncogenes or tumor-suppressor genes, become fixed in the genome of these malignant clones. Further genetic alterations are acquired and as they grant selective advantage, and also become fixed into the genome. These mutations ultimately allow tumor cells to invade *in situ* then to metastasize to other organs (reviewed in (83)). During melanoma progression, transformed melanocytes down-regulate the expression of E-cadherin, as this molecule is critical for skin homeostasis and regulates the cross-talk between melanocytes and keratinocytes. This cross-talk, mediated by E-cadherin and integrins, promotes a cell-to-cell contact that coordinates proliferation (Figure 1.4) (84, 85).

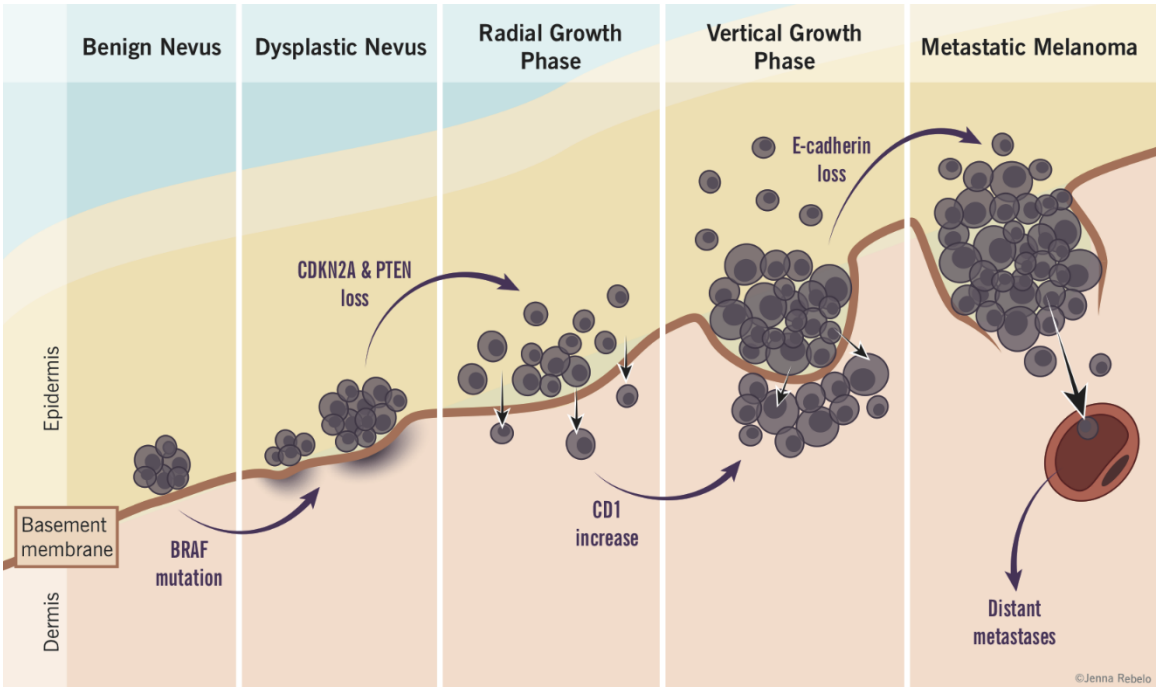

Figure 1.4: Illustration of the melanoma tumor progression.

Melanocytes are located at the basement membrane and contact with surrounding keratinocytes as a homeostatic relationship (not depicted). Melanocytes acquire several mutations, such as in the *BRAF* gene, which are present in about 50% of melanoma cases, and the *PTEN* gene. These mutations confer survival and/or growth advantage over other melanocytes. Then, the malignant clones begin to proliferate and may invade the dermis (radial growth phase). Next, tumor cells massively invade the local tissues in a downward manner (vertical growth phase) and lose expression of E-cadherin, which mediates melanocyte-keratinocyte interaction. Finally, melanoma cells may reach lymphatic vessels and metastasize to regional lymph nodes. Used with permission from Jenna Rebelo, Justine Seuradge, Sultan Chaudhry, and Eric Wong. McMaster Pathophysiology Review, www.pathophys.org.

1.1.2 Murine models of melanoma

Traditionally, the melanoma behaviour and its basic characteristics were studied *in vitro* with two-dimensional adherent cell culture assay. More recently, assays such as the three-dimensional spheroid model allowed the recapitulation of the « tumor organ », by co-culturing melanoma cells and stroma cells in a collagen gel matrix which further mirror the

oxygen and nutrient gradient found in tumor tissues (86, 87). Yet, as we are focused on the molecular understandings of the melanoma immunology, *in vitro* models have a limited application for our research.

Mice remain the premier mammalian model for human biomedical research, due to their availability, ease of manipulation and the excellent knowledge about their genetics. As mentioned above, there is clearly a need for new therapeutics against melanoma. *In vivo* murine models of melanoma are crucial to replicate the mechanisms of melanomagenesis, including initiation, progression and invasion in a natural microenvironment. Indeed, they recapitulate and provide a relevant tumor microenvironment that includes blood vessels, host immune cells and extracellular matrix. Therefore, mice are crucial to study the concept of melanoma immunosurveillance and are clinically relevant, in order to develop novel therapeutic strategies (88).

However, although instrumental for the study of human melanoma, it is important to bear in mind that humans and mice somewhat differ in terms of skin biology, which could impact the modelling of melanoma, in terms of histopathological appearance. For example, there are inherent differences in melanocytes biology: while human melanocytes are located mostly at the basal layer of the epidermis as aforementioned, murine melanocytes are mostly located in the dermis, near the hair follicle (Figure 1.5). Some murine melanocytes are found in the epidermis of hairless areas of the mice skin, for example on the tail and ears. As the dermis is thought to be less supportive for melanomagenesis and protects the melanocyte's DNA from photo-damage, this probably explains why mouse are resistant to UV-induced melanomagenesis (70). Traditionally, shaved mice were exposed to different amounts of UV radiation (high dose and low dose) or to chemical carcinogen such as 7,12-dimethylbenz[a]anthracene (DMBA) to simulate human melanoma. However, these models often lead to other types of skin cancer, for example squamous cell carcinoma and are not relied on nowadays (89, 90). Current experimental systems are vastly superior and resemble greatly the human molecular pathogenesis.

There are three types of murine models to study melanomas: genetically-engineered

models, xenografts and syngeneic transplants models (91). Their relevance depends on the research focus and how closely they replicate genetic alterations, histology and metastatic potential.

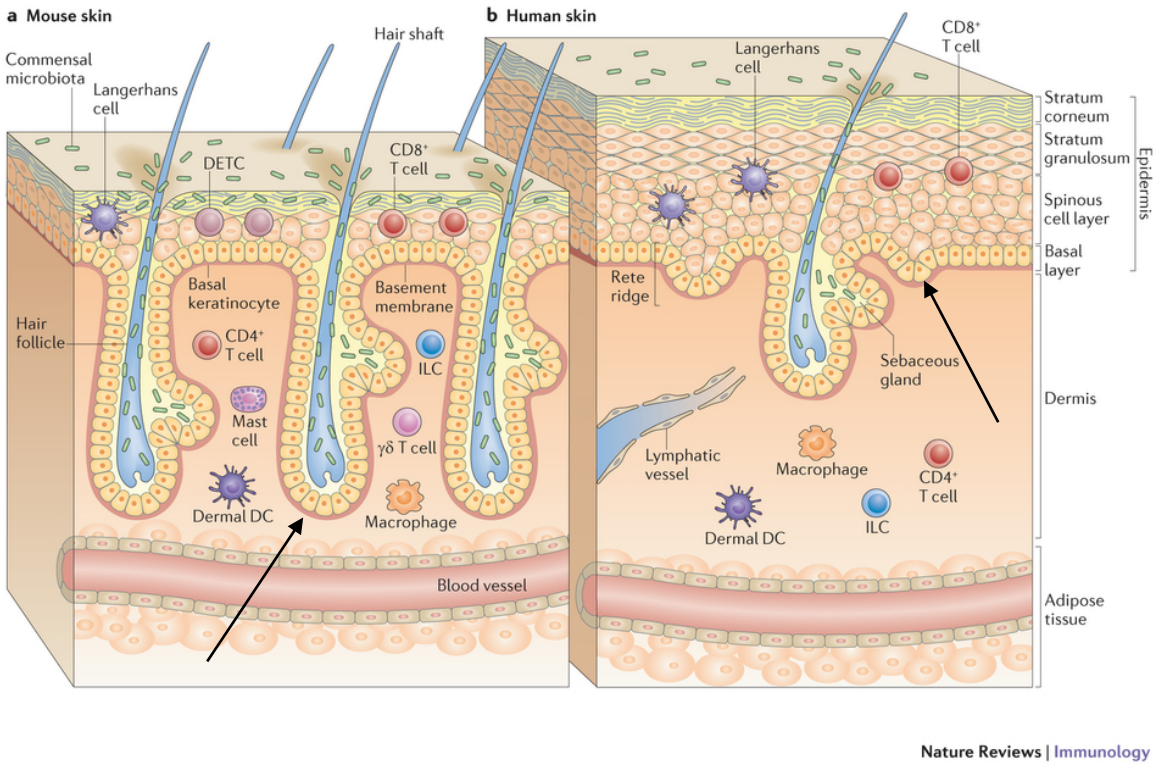

Figure 1.5: Physiological differences in skin between mouse and human.

The arrows indicate where melanocytes are normally located in the mouse and human skin. In the mice, the melanocytes are found near the hair bulb, into the dermis. Alternatively, in humans, they are found at the basal layer of the skin. Another important biological discrepancy is the reduced thickness of the mouse skin, compared to human skin. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Pasparakis, M., et al. (2014). "Mechanisms regulating skin immunity and inflammation." Nat Rev Immunol 14(5): 289-301.), copyright (2016).

Genetically-engineered mouse models were developed as several genomic loci have been identified as implicated in the development of sporadic and familial melanoma and

reproduced into the mice (92). They include different techniques to alter the genome and to study the effect of genetic alterations on the disease formation and progression. For example, gene knockouts, the transgenic and inducible gene expression technology may be used, and can further be tissue-specific and may rely on inducible promoters. The genetically-engineered mice can further be exposed to UV irradiation or chemical carcinogens (93).

Xenograft models consist of the transplant of human melanoma cell lines or patient-derived tumor xenograft into immune-deficient mice, that do not reject human cells. Immunocompromised mice used for this model may lack T lymphocytes, for example the nude athymic (nu/nu) mouse, or may lack both T and B lymphocytes, for example the severe combined immune-deficient (SCID/SCID) mouse (94). In this model, human melanoma cells are permitted to establish and interact directly with the vasculature and the extracellular matrix. Human melanoma cell lines, passaged for several years in a non-physiological milieu, poorly represent original human tumors (95). On the opposite, patient-derived tumor xenografts preserve the clinical characteristics of human tumors, including the genetic mutations, the transcriptome and histopathology (96). However, a major drawback of xenograft models is that the graft does not grow into its natural tumor microenvironment, as the tumor stroma is highly influenced by immune cells that are lacking in these mice (97).

Syngeneic transplant models come in handy when the objective is to study melanoma cells in their adequate microenvironment. Indeed, these models consist of transplanting syngeneic melanoma cells into an immunocompetent mouse recipient that is from the same species, and thus, from the same genetic background. Different mice strains for which their immunophenotype are thoroughly characterized have their counterpart melanoma cell lines developed. Although the cell lines and their sublines express a wide degree of variability in terms of tumor growth rate and metastasis potential, these characteristics are generally well outlined. The use of cell lines permits reproducibility across experiments as the number of passaged cells injected may be held constant (98). Moreover, cellular populations infiltrating the tumor accurately resemble what is seen in melanoma patients

(99). However, as for any other murine model, one model cannot replicate all types of human melanomas (93).

As our laboratory focuses on the cellular mechanisms of antigen presentation via MHC class II molecules, it was evident that we would study the impact of MARCH1 with a model that presented an intact tumor microenvironment, as this factor is critical for tumor progression. Therefore, as our MARCH1-deficient mice have C57BL/6 genetic background, we chose the B16 melanoma model, which is the corresponding syngeneic model.

1.1.3 B16 melanoma model

The B16 melanoma model is the most widely used syngeneic model of melanoma in mice. It is a cell line that originated spontaneously in a C57BL/6 mouse. This cell line, originally described in 1954 by Fidler and his colleagues, has been established *in vitro* after many passages into syngeneic recipients and thus, many sublines derived *in vivo* and *in vitro* exist and differ by their metastatic potential and organ tropism (100). The most common sublines are B16.F10, a highly aggressive variant of the B16.F1 parental line, that metastasize to the lungs after subcutaneous or intravenous inoculation, and B16.F0, referred as poorly metastatic (100). The metastatic potential of the sublines are believed to be due to unique properties of the tumor cells: highly metastatic cells express elevated levels of proteases and glycosidases, which increases their proteolytic and fibrinolytic activities required to invade tissues (101).

The B16 cell line is characterized as poorly immunogenic, as vaccination with irradiated B16 cells in conjunction with adjuvants such as Bacillus Calmette–Guérin (BCG) or *Corynebacterium parvum*, failed to induce immunization to B16 challenge (102). Additionally, the B16 melanoma model is relatively resistant to systemic, high dose, IL-2 treatment, which normally enhances strong anti-tumor immunity with other tumor models (102). Also, B16 cells express very low levels of MHC class I (H-2K^{b}, H-2D^{b}) and II (I-A^{b}) and low or absent levels of co-stimulatory molecules such as CD40L, CD48 and CD54. However, upon *in vitro* treatment of IFN-γ, these cells start expressing both MHC class I

and class II (103). *In vivo*, this cytokine may be produced by both CD8+ and CD4+ T cells and act on melanoma cells in the vicinity, which could augment their recognition by CTLs (102).

Traditionally, as many tumors express MHC class I and as *in vitro* assays demonstrated contact-dependent lysis of tumor cells by tumor-specific, MHC class I-restricted CD8+ T cells, these cells were the choice of interest in early immunotherapies (104, 105). On the other hand, CD4+ T cells were depicted as helper of the CD8 response, by priming cytotoxic T lymphocytes and by supplying cytokines, such as IL-2 (106). This was further supported by the fact that most non-hematopoietic tumors do not express MHC class II (107), thus cannot be recognized by these cells. Therefore, as CD4+ T cells are MHC class II-restricted and could not possibly directly recognize and eliminate tumors cells, CD8+ T cells were associated with a dominant role in tumor immunity.

Unforeseen results by Hung *et al*. (108) reversed the classical doctrine: they demonstrated that CD4+ T cells actually have a major role in the eradication of B16 cells. They vaccinated both CD4- and CD8-deficient mice with irradiated, GM-CSF-producing B16 cells, then, challenged them two weeks later with wild-type B16 cells. The local production of GM-CSF by B16 cells during the primary response lead to an increased activation of bone marrow-derived APCs that could present antigens to both CD4+ and CD8+ T cells. None of the vaccinated CD4 KO mice were able to reject the B16 cells upon challenge, while a significant proportion of the vaccinated CD8 KO mice could reject the tumor challenge successfully. Thus, this suggests that CD8+ T cells alone have a small role in the eradication of B16 cells. They highlighted the critical role of APCs activated by GM-CSF in the priming of *de novo* T cells and during the amplification of the response by presenting antigens to memory CD4+ T cells. Furthermore, the vaccination with B16-GM-CSF cells generated both T_{H1} and T_{H2} prototypical cytokines, IFN-**γ** and IL-4 respectively, and that both were necessary. The release of IFN-**γ** and IL-4 by CD4+ T cells locally activates macrophages, the effectors of the T_{H1} response, and eosinophils, the effectors of the T_{H2} response, as both do not have antigen-specificity. The binary T_{H1}/T_{H2} response was maintained throughout the duration of the experiment, which is surprising considering that

there is generally a commitment to either pathway during a disease, as both cytokines are antagonists and inhibit each other (108).

Therefore, as demonstrated by Hung *et al*. and others, CD4+ T cells can have tumoricidal activity, independently of CD8+ T cells (108, 109) and actually more efficiently than CD8+ T cells (110). They showed that the recognition of tumor cells by CD4+ T cells is indirect and dependent on APCs infiltrating the tumor microenvironment. As such, naive CD4+ T cells are primed in the draining lymph nodes and then migrate to the tumor site. There, they interact with APCs that express high levels of MHC class II and that capture and process antigens shed by dead tumor cells. Thus, these events are highly dependent on the host expression of MHC class II but not on the tumor cell's expression (111). Furthermore, it has been shown that the effector phase of the CD4 response requires the expression of the IFN-**γ** receptor (IFN-**γ**R) by host cells, but not tumor cells (109). IFN-**γ** is a cytokine that has important biological activity, as its receptor is expressed on most cell types and can induce the expression of MHC class II (112). Although not the preferred mechanism, but not excluded, IFN-**γ** produced *in vivo* by CD4+ T cells could induce the expression of MHC class II on tumor cells and augment their recognition. Furthermore, IFN-**γ** might have intrinsic cytotoxic activity on tumor cells, in combination with TNF-**α**. However, the preferred mechanism of the mechanism of action of IFN-**γ** suggests that its activity would be mediated on host cells, both hematopoietic and non-hematopoietic. Indeed, IFN-**γ** can act on macrophages to induce anti-tumor properties, such as the secretion of TNF-**α** and nitric oxide, which leads to malignant cell killing (113, 114). Also, IFN-**γ** can act on endothelial cells part of the tumor stroma, to promote the release of anti-angiogenic factors (105). In short, a much broader role for CD4+ T cells in anti-tumor immunity have been revealed. However, it must be kept in mind that both CD4+ and CD8+ T cell arms are required to orchestrate an optimal anti-tumor immunity.

Finally, the B16 model has many similarities with human melanoma: human melanoma express five different associated antigens, which are all expressed by B16 cells and human melanoma cells express low levels of MHC class I, as B16 cells. However, B16 cells are highly aggressive and rapidly growing. While the mice die weeks following inoculation,

human may survive for months to years after the diagnosis of melanoma. Yet, the B16 model is a reasonable model for human melanoma (102).

1.2 Hypotheses and objectives

While the role of CD4+ T cells in cancer eradication has been demonstrated with massive clinical and experimental data, the mechanisms of tumor immunosurveillance remain to be fully elucidated. Current hypotheses feature infiltrating DCs as key players of tumor immunosurveillance, by presenting tumor antigens via their MHC class II molecule to CD4+ T cells at the proximal lymph node. Our group and others have established that MARCH1 is a significant regulator of DC activation, by ubiquitinating the cytoplasmic tail of MHC class II molecules, which stabilizes their surface expression. The MARCH1-deficient mice were characterized over the years by Satoshi Ishido. It presents a complete loss of MHC class II ubiquitination and thus, stabilized peptide-MHC class II surface expression on its DCs. While other groups assumed that this would enhance the ability of DCs to activate CD4+ T cells, he demonstrated in 2014 that DCs from this mouse are functionally impaired and this limits their capacity to stimulate naive CD4+ T cells (123).

Hypothesis 1

If the loss of MHC class II ubiquitination impedes the immune response, as shown by the MARCH1 KO mice, then we hypothesize that MARCH1-deficient mice will present larger tumors than their wild-type counterpart.

Objective 1

To follow the development of B16 tumors in the MARCH1 KO and its wild-type counterpart.

Hypothesis 2

If *in vitro* studies demonstrated that the functionally impaired DCs from the MARCH1 KO mice prevent the efficient activation of CD4+ T cells, then we hypothesize that the MARCH1 KO mice will present a reduced CD4+ T cell infiltration of the tumors.

Objective 2

To compare both the tumor-infiltrating DC populations and CD4+ T cells in the MARCH1 KO and wild-type mice.

CHAPTER 2: MATERIALS AND METHODS

2.1 Mice and subcutaneous transplantation of B16-F10 tumor cells

2.1.1 Mice

MARCH1 KO mice and their wild-type (C57BL/6) littermate controls were used, the latter in order to limit genetic and environmental variations that may impact the model (116). Both genotypes are bred in the University's animal facilities. Females aged 6 to 8 weeks (aged-matched) were used in accordance with University of Montreal's Institutional Animal Care and Use Committee guidelines.

2.1.2 B16-F10 cell culture

The B16-F10 cell line, kindly donated by Étienne Gagnon, was incubated at 37°C and cultured in complete medium, that is, Dulbecco's Modified Eagle Medium (DMEM, Wisent), supplemented with 10% fetal bovine serum (FBS, Wisent). The medium was replaced and cells were sub-cultivated 1:10 every 2 or 3 days.

2.1.3 Subcutaneous injection

B16-F10 cells were transplanted in mice when they reached ≤ 50% confluence and only if viability exceeded 90%, in order to maximize tumor take. The cells were detached with Trypsin-EDTA (0.25%, Wisent) then rinsed with complete medium. The cells were centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in ice-cold Hanks' balanced salt solution (HBSS) at a concentration of 1 X 10^6 cells/ml. The cell suspension was passed through a disposable cell strainer to remove any cell clumps. 1-ml syringes with attached 27½-G needle were filled with the cell suspension in sterile conditions before moving to the animal facility. There, the mice were randomized and anaesthetized with Isoflurane (Baxter), and injected subcutaneously into the lateral flank with 100 µl of the cell suspension. The last 0,5 ml of the syringe was always discarded as tumor cells tend to collect by the plunger of the syringe, which augments the cell concentration and may impact the results. If no clear « bleb » appeared after the injection, the animals were sacrificed immediately. Tumor growth was measured every second or third days with callipers. Mice were sacrificed after 14 days post-injection.

2.2 Flow cytometry analysis

2.2.1 Isolation of immune cells from tissues

After the sacrifice, the tumors, axillary and inguinal lymph nodes were harvested. The tumors were surgically excised and minced with scissors. 5 ml of dissociation solution (RPMI medium supplemented with 10% FBS, Collagenase type IV (200 U/ml) and DNase I (100 μg/ml)) was added to the tissue for 30 minutes at 37°C with gentle agitation. The inguinal and axillary LNs were isolated and digested in 400 U/mL of collagenase D (Roche) in RPMI-1640 for 30 min at 37°C. After the incubation, the cell suspension was passed through a 70μm cell strainer and washed twice. Inflammatory cells were pre-enriched from the tumors using 80%-40% density gradient centrifugation (Percoll, Sigma-Aldrich).

2.2.2 Staining of TILs and lymphocytes from draining lymph nodes

To avoid non-specific Fc-mediated interactions, the cells we pre-incubated with mouse FcR blocking reagent for approximately 20 minutes on ice, before the staining. Several μL from the cell suspension were aliquoted into a 96-well U-bottom microtiter plate. The anti-mouse antibodies used for FACS analysis were purchased from BioLegend, unless indicated otherwise. The antibody cocktail to stain murine CD3+ T lymphocytes and their activation level was: PE/Cy7 anti-mouse CD3 (1:300, 17A2 clone), PE anti-mouse CD44 (1:200, 1M7 clone, BD Pharmingen), PerCP anti-mouse CD4 (1:200, RM4-5 clone), APC anti-mouse CD62L (1:300, MEL-14 clone, BD Pharmingen), BV421 anti-mouse CD45 (1:200, 30-F11) and BV711 anti-mouse CD11b (1:1000, M1/70 clone). The staining was performed on ice, protected from light, for approximately 35 minutes. All samples were acquired using FACSCanto II (BD Biosciences) and analyzed with FlowJo (Tree Star). The data was plotted and statistically evaluated using the GraphPad Prism version 6 software.

2.2.3 Staining of DCs and macrophages from tumors and draining lymph nodes

The staining procedure, flow cytometry and data analyses were performed as described above for TILs. However, a different cocktail of antibodies for detecting DC-specific molecules was used. The antibody cocktail used included: BV421 anti-mouse CD45

(1:200, 30-F11 clone), PE/Cy7 anti-mouse CD11c (1:200, HL3 clone, BD Pharmingen), APC/Cy7 anti-mouse MHC class II (1:2000, M5/114.15.2 clone), FITC anti-mouse Ly6C (1:600, HK1.4 clone), APC anti-mouse CD103 (1:200, M290 clone), PerCP/Cy5.5 anti-mouse CD64 (1:200, X54-5/7.1 clone), BV711 anti-mouse CD11b (1:1000, M1/70 clone), PerCP anti-mouse Ly6G (1:300, 1A8 clone) and PE anti-mouse CD206 (1:300, C068C2 clone).

2.3 Retroviral plasmid production

2.3.1 Plasmids and cloning

The murine MARCH1 cDNA was kindly provided by Satoshi Ishido, then tagged with YFP and cloned into a pcDNA3.1 plasmid, the latter obtained from Daniel Lamarre. The MARCH1-YFP sequence was excised from the pcDNA3.1 vector by an enzymatic digestion by Pme1 for 3 hours at 37°C. The insert DNA was purified by agarose gel (1% agarose, prepared with TAE buffer 1X) migration. The retroviral entry vector (MSCV-IRES-Thy1.1) was obtained from Connie Krawczyk. This plasmid was digested separately with Pme1 for 3 hours at 37°C. The retroviral entry vector was further dephosphorylated with shrimp alkaline phosphatase for one hour at 37°C.

2.3.2 Insertion of MARCH1 insert into MSCV

The insert was ligated into the retroviral vector with the T4 DNA ligase (Invitrogen), according to the manufacturer's protocol, along with the appropriate controls. We followed a ratio of 3:1 (insert: entry vector) to maximize the ligation efficiency, as the ligation is blunt-ended.

2.3.3 Transformation of competent cells

Competent DH5∝ cells were transformed with the ligation mix, including the controls. Briefly, half of the ligation mix was added to an Eppendorf tube containing 50 µl of competent DH5∝ cells. The tubes were kept on ice for 30 minutes, placed at 42°C for 45 seconds and then kept on ice for 2 minutes. After the heat-shock, 500 µl of 2YT broth was added to the tubes and incubated at 37°C with gentle agitation for 1 hour. After the incubation period, the tubes were spun at maximal speed for 30 seconds, and the bacterial

cells were spread on a 2TY-agar plate, supplemented with ampicillin (100 μg/mL, as MSCV-IRES-Thy1.1 plasmid has a resistance gene for ampicillin) and incubated overnight at 37°C.

2.3.4 Selection of bacteria that incorporated the mMARCH1-YFP-MSCV plasmid

From the colonies that grew on the selective plate, 24 were picked with a pipet tip, re-plated on a fresh 2YT-agar plate supplemented with ampicillin (100 μg/mL), as master-plate, and the tips were added to tubes containing 2YT broth (supplemented with ampicillin, 100 μg/mL). The plates were incubated at 37°C overnight, while the tubes were left overnight at 37°C in an agitator. Plasmid DNA mini-preparation were realized with the « EZ-10 Spin Column Plasmid DNA Minipreps Kit » (Bio-Basic, Markham, Ontario, Canada) according to the manufacturer's protocol. Then, a series of restriction digest analysis were made to validate the plasmid construction: the DNA and enzyme mix were loaded onto an agarose gel (1% agarose, prepared with TAE buffer 1X) and the band profile was examined. Finally, three mini-preparation DNA were sent for sequencing (Genomics platform, Institute for research in Immunology and Cancer, Université de Montréal) with a primer that anneals near the multiple cloning site of the plasmid (Forward, MSCV gag: CCT TGA ACC TCC TCG TTC GAC). A single colony was selected as the sequence of the insert was complete and the insert was in the correct orientation. It was seeded into 200 ml of 2YT broth overnight at 37°C in an agitator. Finally, the plasmid DNA was isolated by the « EZ-10 Spin Column Plasmid DNA Maxi preps Kit » (Bio-Basic, Markham, Ontario, Canada) according to the manufacturer's protocol.

2.5 Statistical analysis

Data were expressed as the means ± standard error of the mean. They were analyzed by Student's *t*-test between two groups. P values < 0.05 were deemed statistically significant.

CHAPTER 3: RESULTS

MARCH1-deficient mice present accelerated tumor growth and larger tumors in the B16-F10 melanoma model than their wild-type counterpart.

Extensive biochemical and molecular genetic evidence demonstrates that the tumor development follows a stepwise fashion, and that changes in gene expression patterns are largely responsible for tumor progression. It is widely accepted that tumors arise initially from monoclonal growth, i.e. a single cell acquires pre-neoplastic genetic alterations. Then, the neoplasm commonly becomes polyclonal during tumor progression. At this stage, the tumor presents a wide cellular heterogeneity, resulting from increased genetic instability of cancer cells (117). Similarly, numerous characterizations of the B16-F10 cell line have described it as phenotypically unstable, both *in vitro* and *in vivo*. Indeed, the parent cell line is actually a very heterogeneous cell population, which varies in terms of metastatic potential: some variants may be highly metastatic while others may be tumorigenic but non-metastatic. After the implantation, the early growth of the neoplasm is dependent on the proliferation and death of tumor cells, but also on factors from the tumor micro-environment, which regulates neovascularization and integration into the vasculature (118, 119). The ensuing growth of the tumor relies on the presence of pro-tumorigenic factors in the micro-environment, tissue remodelling and immunological pressures. We are rather interested in these latter events that include the mechanisms of tumor immunosurveillance.

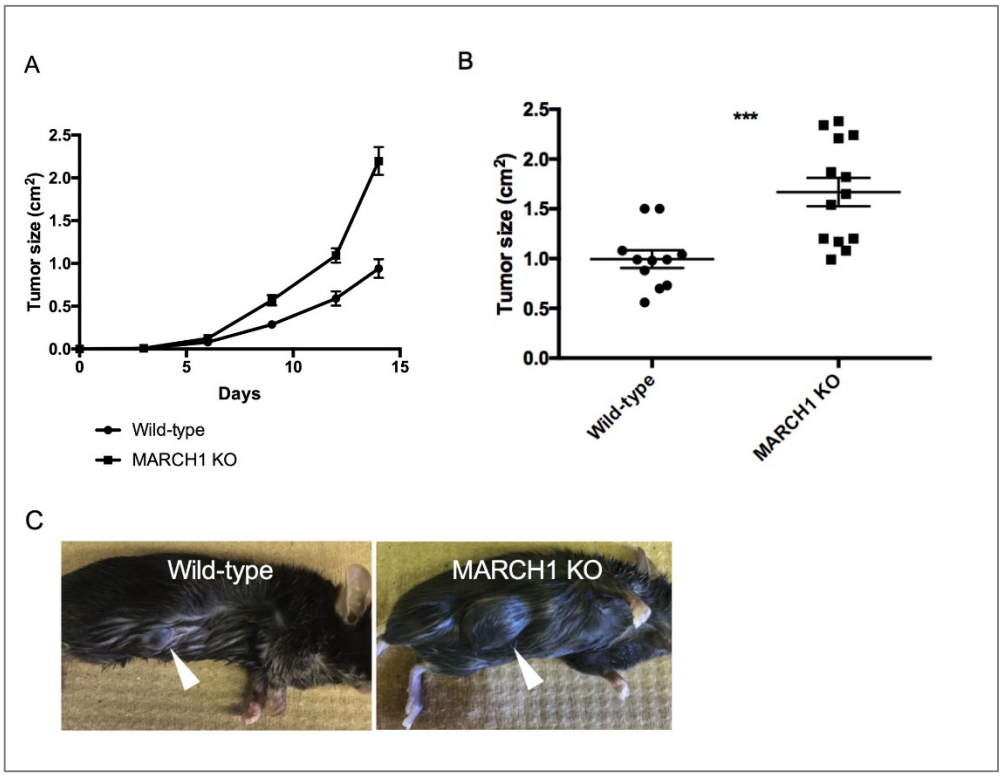

Figure 3.1 MARCH1 KO mice show accelerated B16 melanoma tumor growth and larger tumors than their wild-type littermate counterpart.

To determine the impact of MARCH1 on B16-F10 tumor development, 1×10^5 cells were injected subcutaneously into the lateral flank of MARCH1 KO mice and their wild-type littermate controls. The tumor diameter was measured with callipers every 3 days and at the sacrifice, at day 14 post-injection. The figure depicts a representative of 3 experiments.

A) Tumor growth progression from day 0 to day 14. p= 0.0008 (MARCH1 KO n=3, Wild-type n=3; linear regression)
B) Tumor diameter 14 days post-injection. p=0.0009 (unpaired t-test)
C) Representative photographs of the lateral B16 tumor in wild-type mice and MARCH1 KO mice.

The MARCH1 KO mice present accelerated tumor growth after day 5 (Figure 3.1 A), which resulted in the larger size of the B16 tumor at day 14 (Figure 3.1 B-C). To ensure that our findings were not influenced by tumor take variations from the injection, we sacrificed the mice that did not present a clear bleb under the thin skin after the injection and blinded all experiments. Also, mice that exhibited a tumor diameter less than 0.5 cm^2

after 14 days were not used for further experiments, as we expect that the wild-type mice will develop a 1 cm^2 tumor by day 14 to 21 when successfully injected subcutaneously (102). Several MARCH1 KO mice were euthanized before the end of the model as their tumor became ulcerated before day 14 (not shown).

MARCH1 KO mice present a higher ratio of tumor-infiltrating CD8:CD4 T cells than their wild-type counterpart without significantly different levels of CD4+ T cell activation.

The tumor organ is a complex tissue where reside inter-dependent cell types, including malignant cells, but also stromal cells and infiltrating immune cells. The intra-tumoral cells vary in terms of roles, being either pro-tumorigenic or anti-tumorigenic. Significant experimental and clinical data have suggested that tumor-infiltrating lymphocytes (TILs) are clinically relevant in many different solid cancers and have a significant impact on tumor progression (120). TILs are directly implicated in the concept of cancer immuno-editing, which describes the interplay between cancer cells and immune cells. This concept proposes three steps: immune elimination, immune equilibrium and immune escape. In tumor elimination and equilibrium, certain types of TILs may delay tumor progression (121). While the CD8+ T cells are relatively homogeneous, CD4+ T cells are divided into several subtypes. It seems that it is the ratio between the T cell populations, rather than the extent of the infiltration (122, 123), that is the most predictive of tumor outcome (124).

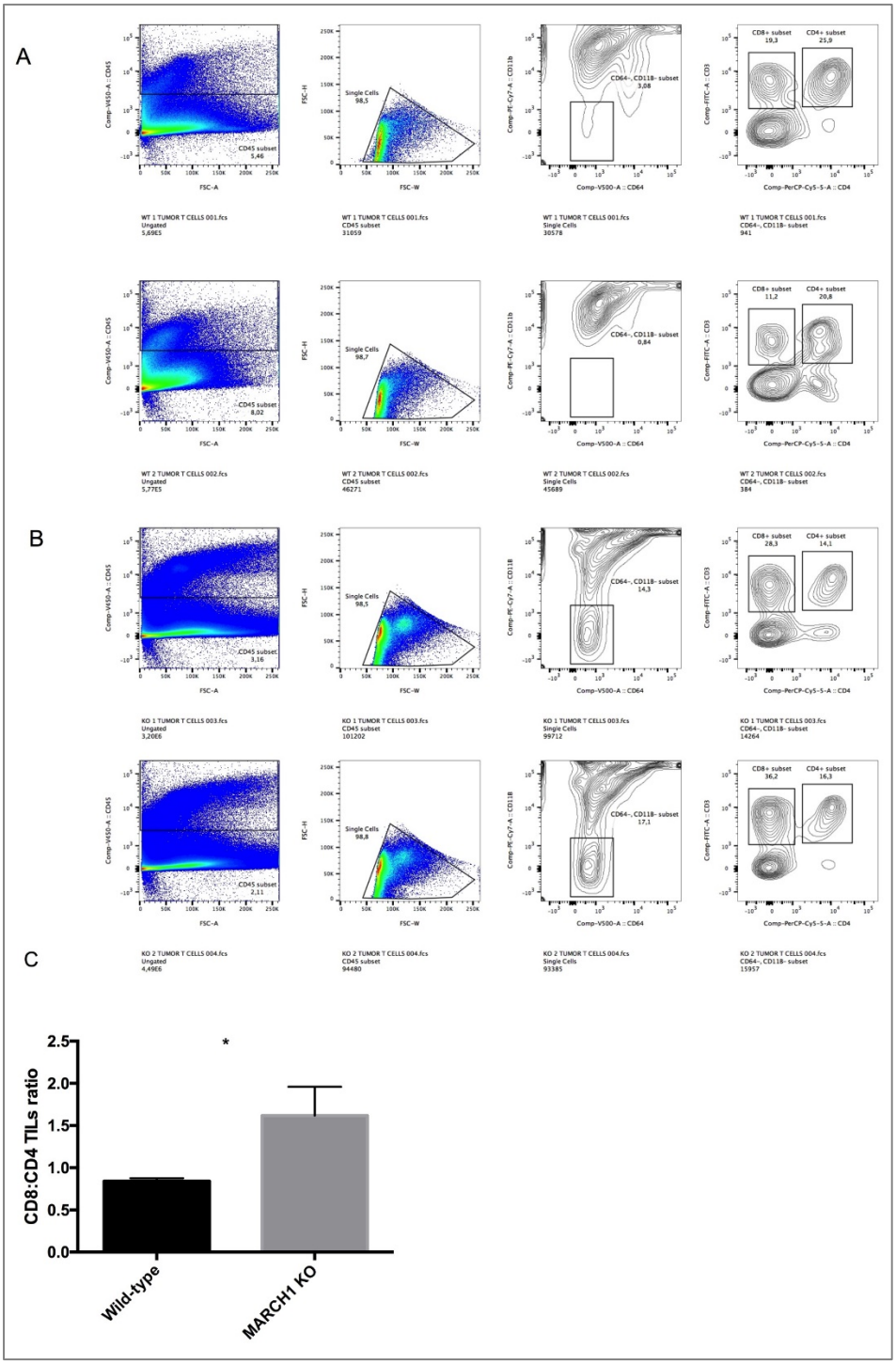

Figure 3.2 MARCH1 KO mice present a higher ratio of tumor-infiltrating CD8:CD4 T cells than their wild-type counterpart.

As TILs have a strong impact on survival, we sought to observe the percentages of infiltrating CD4+ and CD8+ cells in the tumors of MARCH1 KO and wild-type animals.

To gate on TILs, CD45+ cells were gated first. Then, single cells were selected by FSC-H and FSC-W. Next, CD11b+ and CD64+ cells (monocyte) were excluded, as they may express CD4. Finally, as the expression of the co-receptors CD4 and CD8 is mutually exclusive, CD3+ cells were selected and CD4- cells are assumed to correspond to CD8+ T cells. CD3+ CD4+ cells are assumed to correspond to CD4+ T cells. As the tumor size of the MARCH1 KO is larger than the wild-type mice, we expect to observe higher numbers of these tumor-infiltrating immune cells, including TILs. Here we show that the tumors of MARCH1 KO mice are largely infiltrated by CD11b- and CD64- cells. TILs from the MARCH1 KO are dominantly CD8+ T cells, rather than CD4+ T cells. The contrary, a higher proportion of CD4+ T cells, is seen in the wild-type mice. The figure depicts a representative of 2 experiments.

A) Gating strategy and relative percentages of CD4+ and CD8+ TILs in the wild-type mice.
B) Gating strategy and relative percentages of CD4+ and CD8+ TILs in the MARCH1 KO mice.
C) CD8:CD4 TILs ratio in the MARCH1 KO and its wild-type counterpart. p= 0,0219 (MARCH1 KO n=4, Wild-type n=6; unpaired t-test)

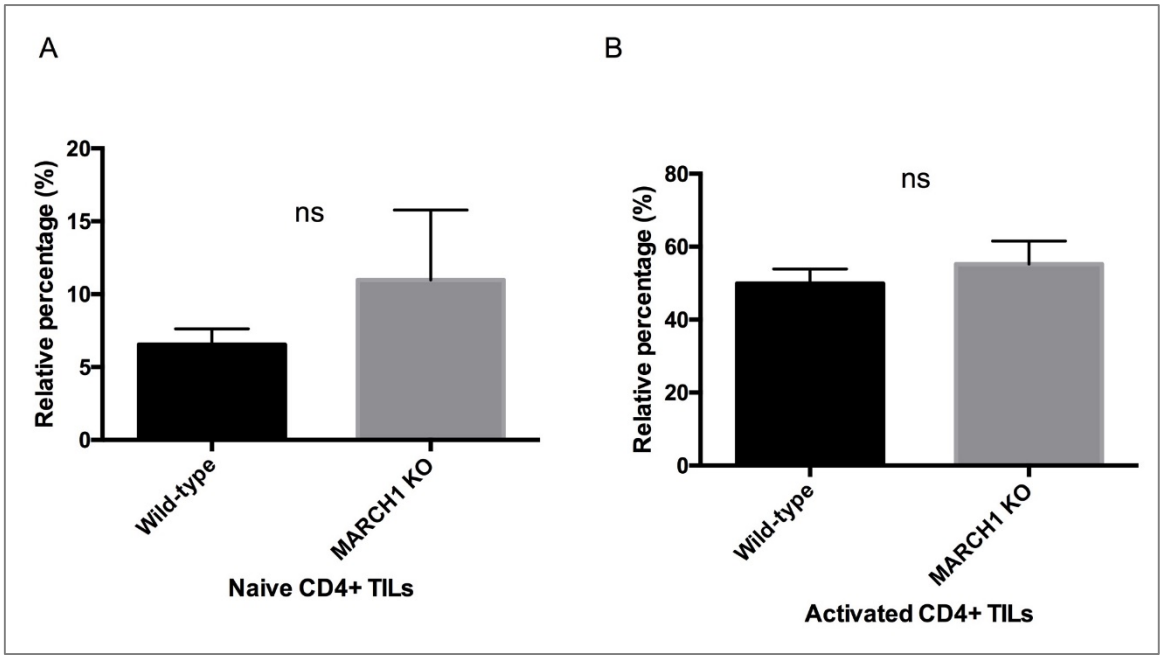

Figure 3.3 No significant differences between the activation levels of CD4+ TILs between the MARCH1 KO mice and its wild-type counterpart.

CD4+ TILs were gated as in Figure 3.2 and were investigated for CD62L and CD44 expression. Naive T cells were characterized as $CD62L^{high}$ and $CD44^{low}$ while activated T

cells were characterized as $CD62L^{low}$ and $CD44^{int\text{-}to\text{-}high}$. Here we show that there is no significant difference in the naive and activated CD4+ TIL population between the MARCH1 KO mice and its wild-type counterpart. The results are from a representative of 2 experiments. (MARCH1 KO n=3, Wild-type n=3; unpaired *t*-test)

A) Relative percentages of naive CD4+ TILs in the wild-type and MARCH1 KO mice.
B) Relative percentages of activated CD4+ TILs in the wild-type and MARCH1 KO mice.

MARCH1 KO mice present similar CD8:CD4 T cells ratios in the tumor-draining lymph nodes.

The tumor-draining lymph nodes (TDLNs) participate actively to the disease progression. It is the site that supports adaptive immune cell priming. Cytokines and tumor-derived antigens can be drained from the tumor to the lymph node, where an increased proportion of DCs are located (125, 126). Finally, the induction of an effective TIL response relies on many factors from the micro-environment and the TDLN, including cytokines and chemokines that attract different cell types, the availability of nutrients and oxygen, but also on the presence of danger signals.

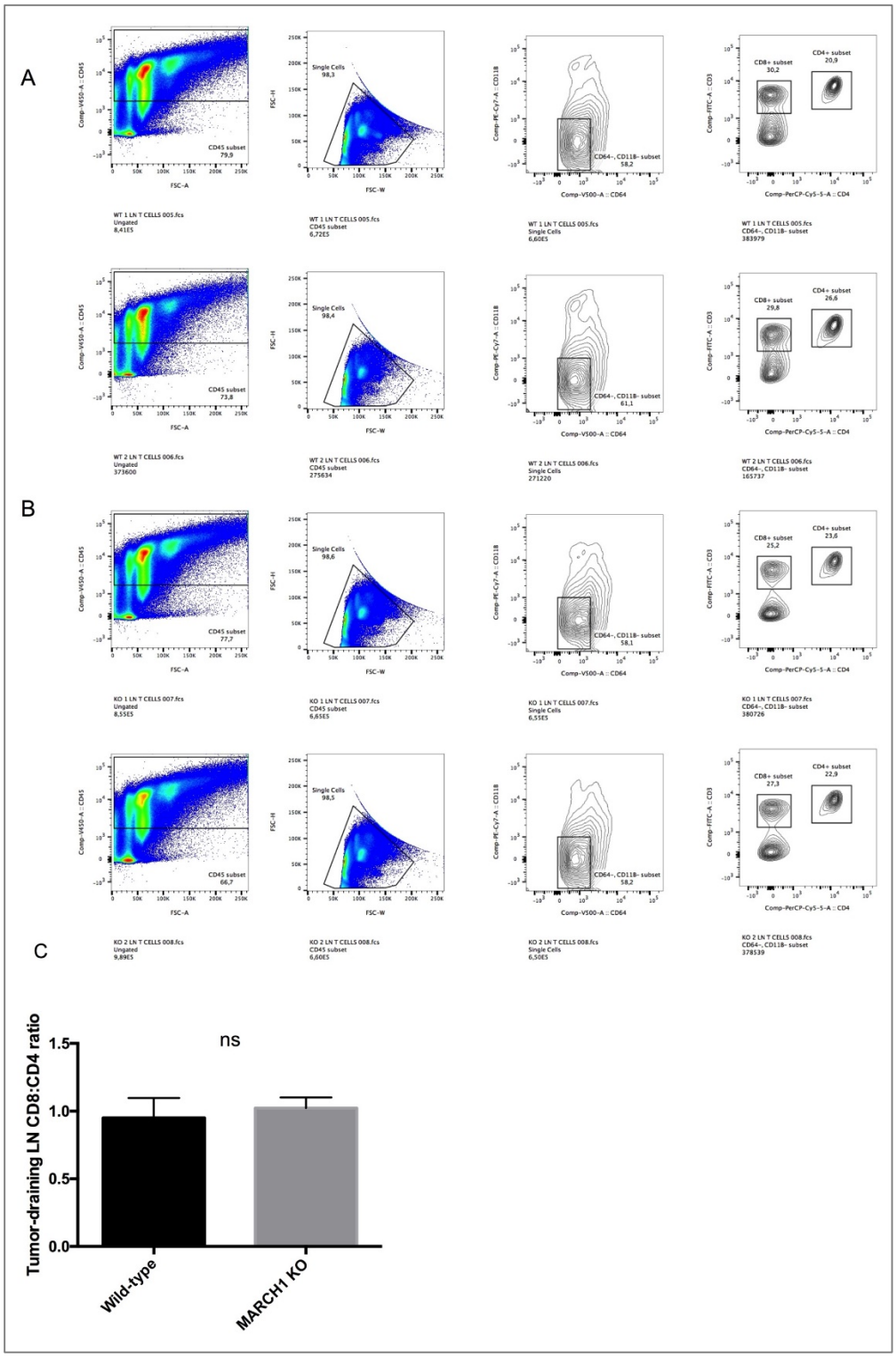

Figure 3.4 MARCH1 KO mice present similar CD8:CD4 T cells ratios in the draining lymph nodes.

As the B16 tumor cells were implanted into the lateral flank of the mice, both the axillary

and inguinal lymph nodes were removed and the ratio of CD8:CD4 T cells was examined by flow cytometry. To gate on CD3+ lymphocytes, a first CD45 gating was performed. Then, single cells were selected by FSC-H and FSC-W exclusion. Next, CD11b+ and CD64+ cells were excluded. CD3+ CD4- cells are assumed to correspond to CD8+ T cells. CD3+ CD4+ T cells were selected by using CD3- and CD4-specific antibodies. Here we show that the ratio of CD8: CD4 T cells are conserved in the TDLNs of MARCH1 KO and wild-type mice.

A) Gating strategy and relative percentages of LN CD4+ and CD8+ lymphocytes in the wild-type mice.
B) Gating strategy and relative percentages of LN CD4+ and CD8+ lymphocytes in the MARCH1 KO mice.
C) LN-infiltrating CD8:CD4 lymphocytes ratio in the wild-type and the MARCH1 KO mice. p= 0,3988 (MARCH1 KO n=4; Wild-type n=6; unpaired t-test)

MARCH1 KO mice present altered melanoma-infiltrating pDC population but not CD103$^+$ CD11b$^-$ cDC population compared to their wild-type counterpart.

As mentioned, the tumor micro-environment is rich in infiltrating immune cells, so we pursued to depict a more inclusive picture of melanoma-infiltrating cells. As DCs are crucial professional APCs and dictate T cell activation and differentiation, we sought to identify and characterize tumor-infiltrating DCs (TIDCs). Melanoma-infiltrating DCs are seen in great diversity, but also with the highest frequencies within the B16 melanoma model, compared to xenograft models and genetically-engineered mouse models (99). Under steady-state conditions, mouse DCs may be divided into plasmacytoid DCs (pDCs) and conventional DCs (cDCs) (127). Under inflammatory conditions, it is highly complex to identify each DC subset, as environmental cues and maturation level may affect the surface expression of markers and because certain markers are shared between DC subsets, even at steady-state. In the B16 melanoma model, CD103$^+$ CD11b$^-$ and CD8α+ conventional DCs can be found (99). These cells are CD11c^{high}, MHC class II^{high}, CD11b-, CD8α$^+$, CD205$^+$ and often CD103$^+$ (128). Also, very few pDCs can also be found infiltrating the tumor (99). They are characterized by CD11c^{int}, MHC class II^{int}, PDCA-1$^+$, LY6C^{high}, CD103$^-$ and CD11b$^-$ (129). Dermal DCs (dDCs), which include Langerhans cells, are present in the non-inflamed skin and are not specifically recruited to the tumor (130). Most dDCs subtypes express CD11b$^+$, CD11c^{high} and MHC class II^{high}, as well as different

levels of CD207 (131).

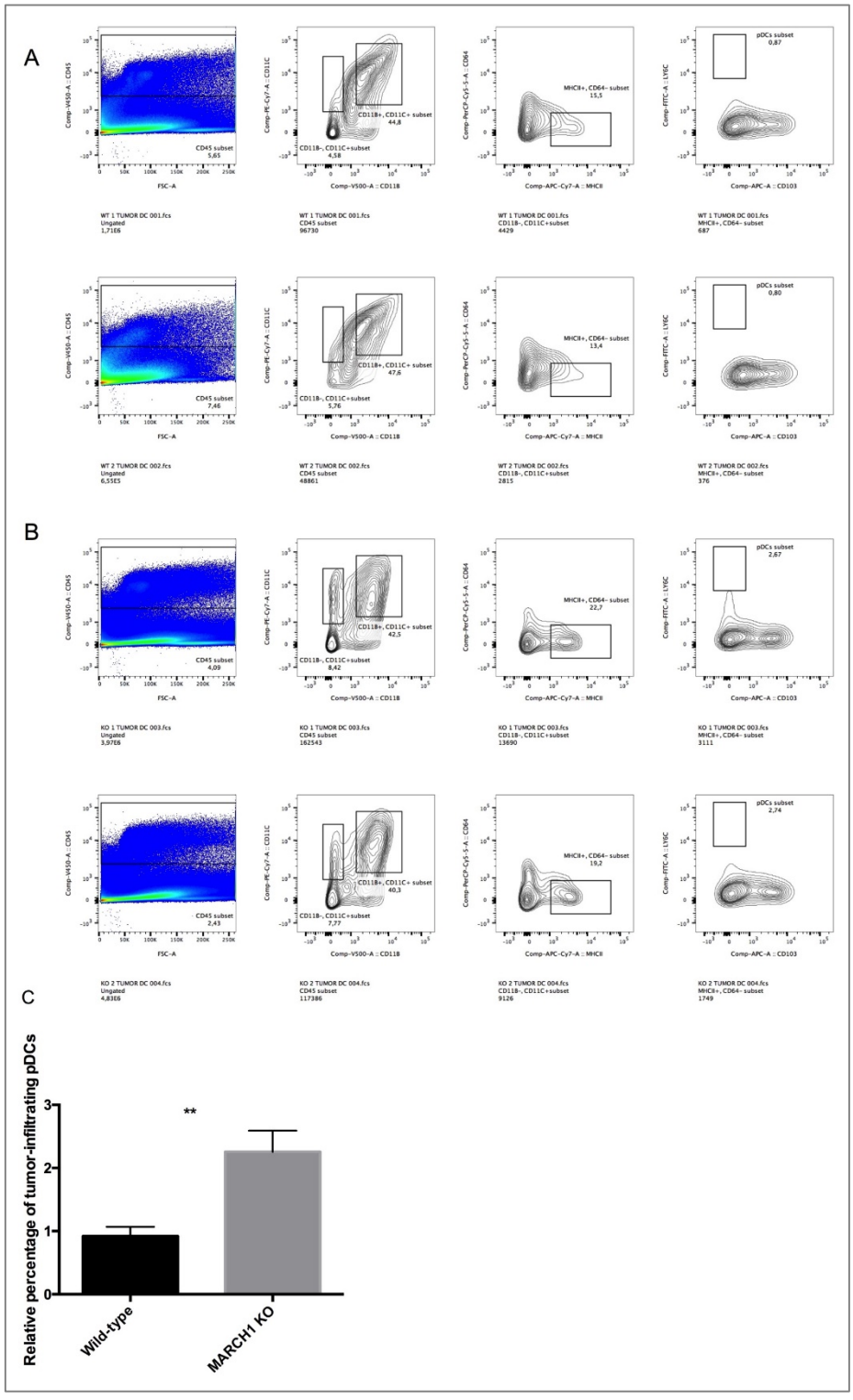

Figure 3.5 MARCH1 KO mice demonstrate significantly increased infiltration of pDCs into the tumor than their wild-type littermate.

To isolate tumor-infiltrating pDCs, an initial CD45 gating was performed on total cells. Then, we gated on $CD11c^{int}$ and $CD11b^{-}$ cells. Next, we excluded $CD64^{+}$ cells and gated on MHC class II^{int} cells. Finally, $CD103^{-}$ and $LY6C^{high}$ (++) were selected and considered as pDCs. We observed that the MARCH1 KO mice demonstrate a large population of $CD11c^{+}$ $CD11b^{-}$ $CD64^{-}$ and MHC class II^{+} population that infiltrated the tumor, compared to the wild-type mice. Also, we observed an increased population of melanoma-infiltrating pDCs in the MARCH1 KO, versus its wild-type counterpart. The figure shows results from a representative of 2 experiments.

A) Gating strategy and relative percentages of tumor-infiltrating pDCs in the wild-type mice.
B) Gating strategy and relative percentages of tumor-infiltrating pDCs in the MARCH1 KO mice.
C) Relative percentage of tumor-infiltrating pDCs in the wild-type mice and the MARCH1 KO mice. p= 0,0033 (MARCH1 KO n=4; Wild-type n=6; unpaired t-test)

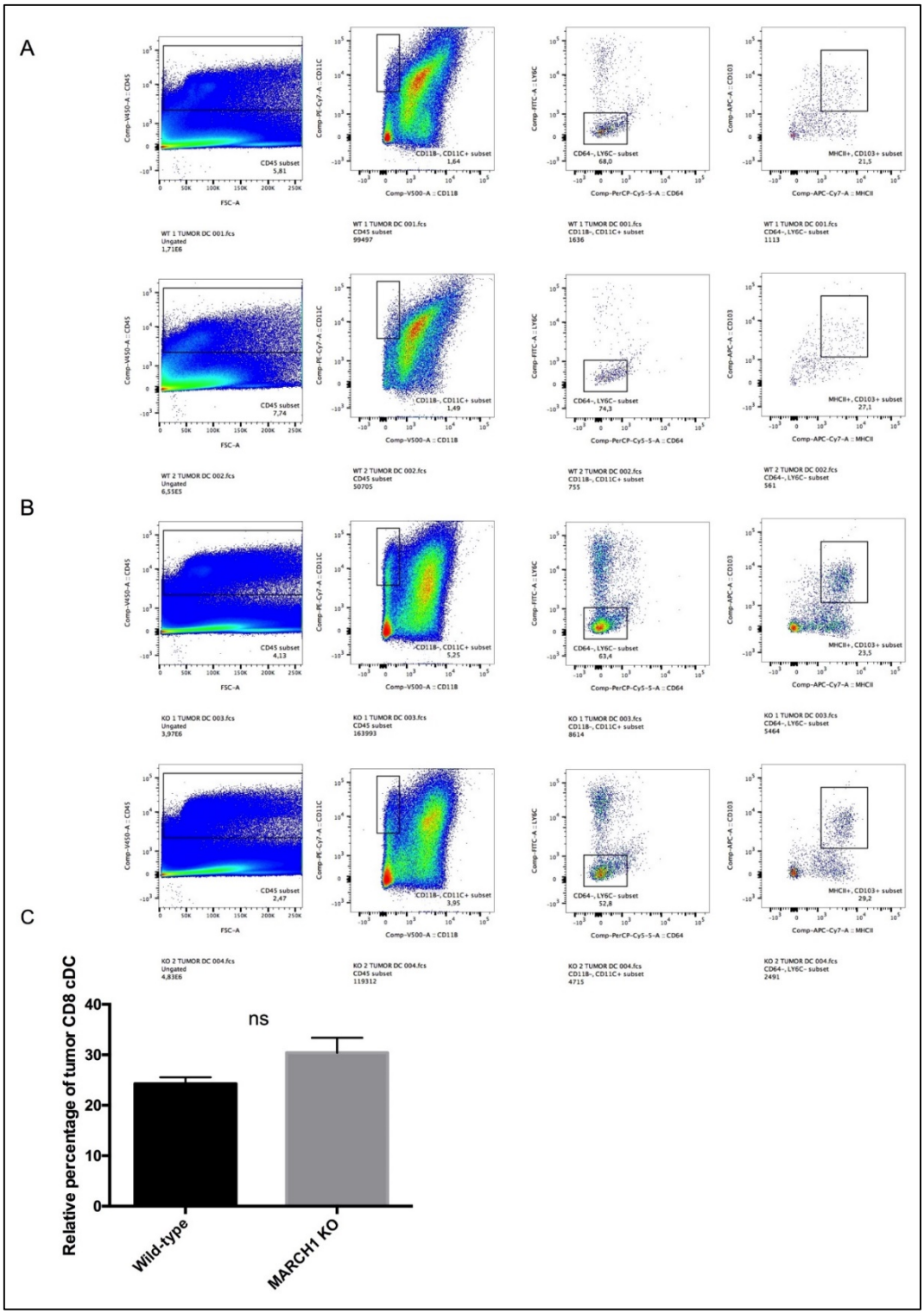

Figure 3.6 MARCH1 KO and wild-type mice demonstrate similar CD103^{+} CD11b^{-} cDC populations infiltrating the tumor.

To isolate tumor-infiltrating $CD103^+$ $CD11b^-$ cDCs, an initial CD45 gating was performed on total cells. Then, we gated on $CD11c^{high}$ and $CD11b^-$ cells. Next, we excluded $Ly6C^+$ $CD64^+$ cells. Finally, $CD103^+$ and MHC class II^+ cells were selected and considered as $CD103^+$ $CD11b^-$ cDCs, even though we did not stain for CD8α. This population could not have been contaminated with Langerhans cells, which populate the epidermis as a homogenous population expressing $CD207^{high}$ $CD11b^{int}$ and $CD103^-$ (132). However, the dermis is populated by four subtypes of dDCs ($CD207^{high}$ $CD11b^{int}$; $CD207^+$, $CD11b^{low}$; $CD207^-$ $CD11b^-$; $CD207^-$ $CD11b^+$), all expressing MHC class II^{high}, which could potentially contaminate the $CD103^+$ $CD11b^-$ cDC population. To circumvent this, we gated on $CD11c^{++}$ as the four subtypes of dDCs express only intermediate levels of CD11c (133). Thus, we could safely conclude that both MARCH1 KO mice and wild-type demonstrated similar populations of $CD103^+$ $CD11b^-$ cDCs that infiltrated the tumor. The results are from a representative of 2 experiments.

A) Gating strategy and relative percentages of tumor-infiltrating $CD103^+$ $CD11b^-$ cDCs in the wild-type mice.
B) Gating strategy and relative percentages of tumor-infiltrating $CD103^+$ $CD11b^-$ cDCs in the MARCH1 KO mice.
C) Relative percentage of tumor-infiltrating $CD103^+$ $CD11b^-$ cDCs in the wild-type mice and the MARCH1 KO mice. p=0.1061 (MARCH1 KO n=4, Wild-type n=6; unpaired t-test)

CHAPTER 4: DISCUSSION

The process of antigen presentation is essential to initiate T cell immunity, both in the context of invading pathogens and malignancy. It requires the formation of a peptide: MHC class II complex at the surface of APCs that will interact with the TCR complex and promote the induction of a specific T cell response. The surface expression of the MHC class II molecules on DCs requires maturation stimuli for the latter. The expression is regulated through ubiquitination of its cytoplasmic tail by the E3 ubiquitin ligase MARCH1. Thus, MARCH1 is a significant regulator of MHC class II-mediated antigen presentation. The maturation of the APC, through TLR signalling, leads to the down-regulation of MARCH1 expression and stabilizes MHC class II surface expression. Furthermore, the loss of MHC class II ubiquitination, such as in the MARCH1-deficient mice, leads to functional impairments of DCs. As MHC class II-restricted antigen recognition by CD4+ T cells is fundamental for anti-tumor immunity, we sought to elucidate the role of MARCH1 in the mouse B16-F10 melanoma model. As the MARCH1 KO mice has a genetic background of a C57BL/6 mice, the injection of B16 melanoma cells into its syngeneic host permits to study the anti-tumor immune response in MARCH1-sufficient cells. Furthermore, this model is poorly immunogenic, as B16 melanoma cells do not express either MHC class I nor MHC class II molecules, which was interesting for our study. Hence, we compared how the tumor progresses in MARCH1-deficient mice after inoculation of the tumor cells *vis-à-vis* their wild-type littermates.

We initially compared the progression of the B16 tumor growth between MARCH1 KO and their wild-type littermates. We found that the subcutaneous injection of B16 melanoma cells in the wild-type mice progressed as indicated the literature, and generated a tumor size of about $1cm^3$ by day 14 (102). As the B16 tumor growth is poorly immunogenic and very aggressive, T cells responses in the wild-type mice results in complete ignorance or tolerance induction. We found that the tumors from MARCH1 KO mice progressed more rapidly upon the B16 challenge than the wild-type mice (Figure 3.1A). This difference was found after day 5 and persisted until the end of the protocol, by day 14. This resulted in larger tumor sizes in the MARCH1 KO mice, compared to the wild-type mice, at the time

of sacrifice (Figure 3.1 B-C). This experiment was reproduced several times, blinded every time, and presented the same results. Although not shown, several MARCH1 KO mice had to be euthanized before day 14 because the mass became ulcerated. As demonstrated by our group and others, the expression of MARCH1 protein is mainly limited to APCs of the secondary lymphoid organs, such as immature DCs. This suggests that MARCH1-deficient mice progress more severely after the tumor transplantation due to decreased or ineffective early immunological pressures compared to the wild-type mice, rather than from impaired implantation of tumor cells into the vasculature. We thus pursued to compare the immune cell populations implicated in the B16 melanoma model.

Tumor infiltrating lymphocytes (TILs) are well-known to have a significant impact on the progression and severity of solid malignancies (124). Therefore, we examined CD3+ T cell populations from the tumors by flow cytometry. We found that the MARCH1-deficient mice presented an increased infiltration of CD3+ T cells in the tumors compared to its wild-type counterpart that correlates with the larger size of the tumor in the MARCH1-KO mice. Although the literature is controversial, it is generally considered that the extent of TILs is predictive of a good prognosis, as it suggests the mounting of a T cell response to tumor antigens in the context of cancer immunoediting. However, more recent studies suggest that it is rather the proportion of different T cell populations that is more important than the extent of infiltration of lymphocytes into the tumor. Therefore, we proceeded to stain the both tumor-infiltrating and TDLNs CD4+ and CD8+ T cell populations. This staining revealed that CD4+ T cells were more abundant than CD8+ T cells in the tumor masses of wild-type mice (Figure 3.2). Conversely, MARCH1 KO mice had a higher ratio of tumor-infiltrating CD8+ T cells to CD4+ T cells. However, we did not observe any significant difference between the wild-type and MARCH1 KO mice in CD4+ and CD8+ T cell ratios in TDLN. The TDLNs are an attractive site for the priming of both CD4+ and CD8+ naive T cells by APCs. Tumor antigens can be drained via the lymphatic vessels and be presented by LN-resident DCs. More effectively, tissue-migrant DCs may capture tumor antigens at the site of inflammation and migrate to the TDLN where they prime naive T cells either by direct presentation or cross-presentation (134). Naive T cells generally circulate through the blood and the lymphatic vessels as they express CD62L and low levels of tissue-

specific adhesion molecule, but can however infiltrate peripheral tissues too. Thompson *et al*. demonstrated that B16 tumor-specific and nonspecific naive T cells can actually infiltrate the melanoma and become activated and differentiated there, independently of the presence of tumor-draining lymph nodes (135). We showed that a small proportion of naive CD4+ T cells are indeed recruited to the tumor, but we did not observe any significant differences between MARCH1 KO and wild-type naive CD4+ TILs. As expected, we also observed a high infiltration of activated CD4+ T cells in the tumor, but without any significant differences between the two genotypes.

As shown by several groups, tumor-infiltrating CD4+ T cells, but not CD8+ T cells, are required for experimental B16 tumor rejection, as they are critical in both the priming and the effector phase of the immune response. As B16 cells do not express MHC class II molecules, CD4+ T cells depend on DCs to present MHC class II-restricted antigens, as shown both *in vivo* and *ex vivo* with antigen-loaded DCs (136, 137). On the other hand, CD8+ T cells play only a small role in B16 tumor eradication, as explained partially by the fact that B16 cells and human melanomas express Fas ligand and are therefore protected from direct killing by CTLs (138). Although it is known that DCs can prime CD4+ and CD8+ T cells and that both are required for optimal response, CD4+ T cells have a dominant role in the development of efficient anti-tumor responses in the case of experimental B16 melanoma. Therefore, this may partially explain why the subcutaneous implantation of B16-F10 cells in the MARCH1 KO mice become larger than the wild-type mice. The skewing to a poorly effective, CD8-dominant T cell response in the MARCH1 KO mice permits a more aggressive proliferation of implanted B16 malignant cells. In contrast, in the wild-type mice, even though spontaneous tumor rejection in a classical B16 experimental model does not occur, the immunological pressures are more efficient to limit the proliferation of malignant cells. This suggests that the immune equilibrium phase, in which there is fine balance between the efficient elimination of tumors cells and the immune-editing of the latter, appears longer in the wild-type mice than in the MARCH1 KO mice. Thereafter, the malignant cells rapidly overcome the immunological pressures and escape the immunological defences.

As CD4+ and CD8+ TILs are dependent on the presentation of tumor antigens by TIDCs, either by direct presentation or cross-presentation, respectively, we sought to compare the melanoma-infiltrating DC populations between the MARCH1 KO mice and its wild-type counterpart. In the B16 melanoma experimental model, cytokines produced by melanoma cells and peri-tumoral cells recruit low levels of pDCs and higher levels of CD103+ CD11b- cDCs.

We found that the MARCH1 KO mice had a significantly increased pDC population recruited to the tumor, in comparison to the wild-type mice. pDCs are not normally found in the non-inflamed skin, as they mostly reside in the blood and secondary lymphoid organs. In primary melanoma, pDCs are rare but may migrate to the tumor site, where they are associated with the establishment of an immunosuppressive milieu that drives tumor growth (139). As pDCs express both TLR-7 and TLR-9, which sense foreign nucleic acids, and thus are potent mediators of the anti-viral immune response, they are not found in an activated state in the melanoma lesion due to a lack of TLR stimulation. Thus, at steady-state, pDCs are described as tolerogenic. Their activation level may be verified with high expression of the marker ICOS and low expression of markers CD80 and CD86, which was not shown in this memoir. Furthermore, melanoma cells directly impede the production of interferons by pDCs by secreting immune-inhibitory molecules such as IL-10, TGF-β and prostaglandin E2 (140). The measure of these cytokines in both the wild-type mice and the MARCH1 KO mice would be interesting for future investigations. Liu *et al*. demonstrated that the presence of activated pDCs in the B16 tumor drove the infiltration of CD8+ T cells to the tumor, which ultimately resulted in a CD8+ T cell-mediated delayed tumor growth (141). This is in line with what we have seen in the MARCH1 KO mice; an increased population of melanoma-infiltrating pDCs and a higher proportion of CD8+ T cells. However, in the case of the MARCH1 KO mice, the disease progressed more aggressively than in the wild-type mice. This contradiction, i.e. the increased tumor infiltration of pDCs and CD8+ T cells that is not correlated with tumor regression in the MARCH1 KO mice, may be explained by the fact that Liu *et al*. injected CpG-activated pDCs directly into an established tumor (day 7). The activated pDCs secreted cytokines drove an early infiltration of NK cells and killing of tumor cells, which

liberated tumor antigens. Those were picked up by endogenous cDCs that were responsible for the cross-priming of CD8+ T cells. On the other hand, the injection of resting pDCs into the tumor did not lead to the recruitment of CD8+ T cells to the tumor nor to delayed tumor growth (similarly to their saline control). In the MARCH1 KO mice, we observed an accumulation of tumor-infiltrating pDCs which coincided with an increased CD8+ T cell infiltration. However, it is unlikely that the tumor microenvironment from the MARCH1 KO mice could activate pDCs as their activation depends on TLR stimulation by exogenous nucleic acids.

We further demonstrated that most DCs that infiltrated the tumors of both MARCH1 KO and wild-type mice expressed CD11c. They appeared to be further divided into $CD11b^{int\text{-}to\text{-}high}$ and CD11b- cDCs. As opposed to pDCs, cDCs are much more potent to capture and process cell-associated antigens and prime T lymphocytes. Specifically, $CD103^{+}$ $CD11b^{-}$ cDCs have been shown to be potent inducers of a T cell-mediated immunity upon maturation. However, we did not observe any significant differences between MARCH1 KO mice and its wild-type counterpart in terms of tumor-infiltrating $CD103^{+}$ $CD11b^{-}$ cDCs. Although we observed a higher number of cDCs in the MARCH1 KO than its wild-type, this is rather a correlation with the tumor size. The percentages were conserved between the two genotypes.

Many studies indicate that the intratumoral inflammatory milieu drives cDC dysfunction by unclear mechanisms, which contributes to tumor evasion. As demonstrated by Gerner and Mescher, in a number of subcutaneous tumor models, such as the B16 experimental model, the tumor microenvironment generates DC dysfunction that leads to CD4+ T cell-mediated ignorance or tolerance (142). They showed that the tumors were infiltrated by a large population of CD11b+ DCs with a partially activated phenotype that closely resemble immature dDCs. In contrast to immature dDCs, the phenotype of CD11b+ cDCs exhibited intermediate levels of MHC class II and co-stimulatory molecules and low expression of IL-12. However, although TIDCs are quite efficient at infiltrating the tumor in high numbers, these cells appeared to be partially defective at exiting the tumor to migrate out to the lymph nodes. Furthermore, TIDCs were less efficient than immature dDCs to

internalize soluble protein antigens. This directly impacted their potential to prime naive CD4+ T cells in the TDLN and to stimulate effector CD4+ TILs, in addition to their partially mature phenotype.

It is important to note that APCs derived from the MARCH1 KO mice express, as expected, high and stabilized MHC class II molecules at their cell surface, which may bias any flow cytometry gating based on MHC class II expression. Indeed, cells that express MHC class II^{low} become MHC class II^{int}, as shown in several papers (29, 115), which may impact the analysis of the MHC class II^{+} populations. This is surely an important limitation in the study of such cell populations, such as in ours'. Therefore, the analyses of MHC class II-expressing cells performed in this memoir were carried out by avoiding gating strategies based on this marker.

The stabilized MHC class II expression on cDCs, as in the MARCH1 KO mice, leads to cell-intrinsic impairments in those cells, as reported by Ishido and his colleagues (29, 115). They demonstrated that although the MARCH1 KO mice does not show reduced percentage of cDC populations, cDCs were less efficient to activate and differentiate naive CD4+ T cells. Secondary lymphoid organ-derived cDC from the MARCH1 KO mice demonstrated significantly reduced surface expression levels of the co-stimulatory molecules CD4 and CD8. Furthermore, *in vitro* studies demonstrated that upon CD40 or LPS stimulation, MARCH1 KO cDCs produced less IL-12 and TNF-α. This was accompanied by poor MHC class II-restricted antigen presentation capacities and thus, diminished potential to activate and differentiate naive CD4+ T cells (29, 115). The impairment of MARCH1-deficient cDCs to activate naive CD4+ T cells might actually explain why we observed less CD4+ TILs in the MARCH1 KO mice. The findings of Ishido were based on ovalbumin (OVA) as a model antigen. As future work, we could inject B16-F10 cells expressing the OVA antigen (B16-OVA) and test the activation of CD4+ T cells with a MHC tetramer assay. As the priming of naive CD4+ T cells in the lymph nodes of MARCH1-deficient mice is less effective than in the wild-type mice, a lower percentage of activated CD4+ T cells migrate to the tumor. Furthermore, as we indirectly assessed CD8+ T cells as CD3+ CD4- cells, their increased proportion seen

infiltrating the tumor might actually be reminiscent of this gating strategy, and not explained by a specific recruitment. Hence, as less CD3+ CD4+ T cells are observed in the tumor mass, it is obvious that proportionally, CD3+ CD4- T cells percentages will augment. Furthermore, as the priming of CD4+ T cells primarily occurs in the TDLN by cDCs, we should not observe any difference between the activation status of CD4+ TILs in the tumors of MARCH1 KO and wild-type mice, as mainly primed T cells migrate to the tumors. This is consistent with what we demonstrated.

Additionally, Oh *et al*., recently showed that defects in MHC class II ubiquitination and specifically MARCH1 deficiency, resulted in severe impairments in the generation of thymic-derived Treg cells and a large reduction in the number of these cells (30). Central tolerance is achieved by thymic DCs, which specifically express MARCH1 unlike TECs, and present antigens to immature thymocytes to differentiate them into Foxp3+ Treg cells. This process was found to depend on MARCH1-mediated MHC class II ubiquitination. In solid tumors, Foxp3+ Treg cells are found to infiltrate the tumor and generate a tolerogenic environment. The relative success of immune check-point inhibitors, in conjunction with inactivation or depletion of Foxp3+ Treg cells, are being studied as they may additionally boost the anti-tumor response (143). However, Foxp3+ T reg cell depletion alone in an established OVA-expressing B16 tumor failed to induce successfully tumor regression as supported by different groups (144, 145), suggesting that this pathway alone is not sufficient and other immunological pathways are required. Yet, as the MARCH1 KO mice are largely depleted in thymic-derived Treg cells, it would be interesting to study how this could influence the development of another syngeneic tumor transplantation model, such as the mouse colon adenocarcinoma (MC38) in C57BL/6 mice. In this model, the depletion of CD4+ CD25+ T cells leads to a complete tumor rejection (146), which suggests that Treg cells have a dominant effect in this model and that the MARCH1 KO may have an advantage over its wild-type counterpart.

Collectively, our findings demonstrate that in the context of subcutaneous B16 melanoma, MARCH1 deficiency impairs the generation of a CD4+ T cell response. As characterized by others (139, 142), this poorly immunogenic tumor transplant model generates an

immunosuppressive microenvironment that drives DC dysfunction. TIDCs exhibit a partially activated phenotype in addition to a limited migration to the lymph nodes, which further impairs the generation of an effective CD4+ T cell response. As shown by Ishido and his colleagues (29, 115), DCs with stabilized peptide: MHC class II expression at their surface, such as DCs isolated from MARCH1 KO mice, suffer from cell-intrinsic defects that prevent them to efficiently prime naive CD4+ T cells. Thus, MARCH1-deficient mice, in addition to the tumor burden which further impacts TIDCs, demonstrate a significantly depleted infiltration of CD4+ T cells in the tumor, compared to their wild-type counterpart. This leads to a rapid tumor evasion from the impaired immune responses and a rapid progression of the disease. Future work should include a cohort of MHC class II knock-in mice, as these mice are likely to demonstrate a phenotype similar to the MARCH1 KO mice, that is high expression of MHC class II at the surface of MHC class II+ cells, without requiring the complete deficiency of the *March1* gene, which results in functional defects of cDCs and a large depletion of Treg cells.

Finally, we developed a retroviral plasmid system via blunt-ended ligation that expresses YFP-tagged murine MARCH1 cDNA that may be further used to produce retroviral particles by Phoenix-Ampho cells in order to transduce hard-to-transfect mammalian cells. The long-term forced expression of murine MARCH1 into dividing immune cells will be a valuable tool for our group. For example, retroviral vectors expressing murine MARCH1 could be used to transduce *ex vivo* MARCH1-deficient dividing immune cells in order to re-introduce its expression and re-establish the wild-type phenotype.

Retroviral vectors are considered as an important tool in stable gene transfer in hard-to-transfect mammalian cells. Retroviral and lentiviral vectors are a plasmid-based technology derived from the genome of the retroviridae family. Both lentiviral and retroviral-mediated transduction lead to a long-term transgene expression in the target cell, as compared to other viral systems (147). The choice of a retroviral or lentiviral vector must be done depending on the research focus, as the biology of the wild type parent viruses varies. As we are seeking a stable expression of the *MARCH1* gene into dividing primary murine cells, but also cell lines, we will develop a retroviral vector that expresses a large

tropism and that will expresses the murine MARCH1 protein. To do so, we will rely on the second generation retrovirus producer cell line, the Phoenix helper-free packaging cell line, which are 293T cells stably carrying two helper constructs encoding for the *env* protein and *gag-pol* proteins. Hence, the experimenter must only transfect the transfer plasmid encoding the gene of interest by calcium-phosphate or lipid-based methods. Thus, via *trans*-complementation, retroviral particles generated by Phoenix cells that are replication-defective, i.e. they can not replicate further in target cells (148). Furthermore, Phoenix-Ampho cells produce retroviral particles that may transduce a wide range of dividing mammalian cell types, including human cells. Accordingly, the Phoenix-Ampho producer cell line is a safe and efficient mean to produce retroviral vectors.

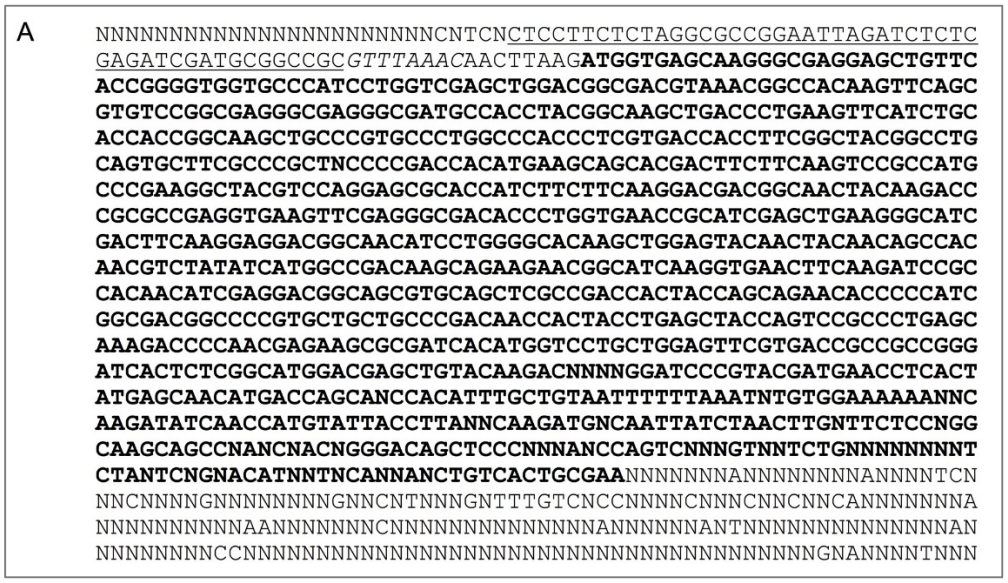

Figure 4.1 Nucleotide sequence of the MSCV-mMARCH1-eYFP tagged retroviral plasmid.

To change the backbone of the MARCH1 cDNA insert, it was excised from the pcDNA3.1 plasmid by a Pme1 digestion. Separately, another Pme1 digestion was performed to open the retroviral entry plasmid MSCV-IRES-Thy1.1. Then, a blunt-end ligation was performed to ligate the insert into the new vector. Competent bacterial cells were transformed with the ligation mix, selected for ampicillin resistance and 24 mini-preps were performed from single isolated colonies. Several restriction digestions were performed to verify the validity of the new construct (not shown). Then, a sample mini-prep susceptible to have inserted the cDNA sequence with the correct orientation was sent

for sequencing. The sequence of the MSCV retroviral plasmid was identified and underlined. The unique sequence of the Pme1 site, as it was not destroyed by the blunt-end cloning, was identified in *italic*. Finally, the sequence (not complete, as a single primer was used for sequencing) of the insert was identified in **bold**.

In summary, we found that mice deficient in the MARCH1 protein develop more aggressive and larger tumors than their wild-type littermates upon the B16 melanoma challenge. This is most probably explained by the fact that DCs from the MARCH1 KO mice express stabilized MHC class II expression at their cell surface, which leads to cell-intrinsic defects that prevent these cells from efficiently priming CD4+ T cells *in vitro*. In line with this, we demonstrated, by using an *in vivo* model of melanoma, that MARCH1 KO mice show significantly reduced tumor-infiltrating CD4+ T cell populations, but not LN infiltrating CD4+ T cell populations, *versus* their wild-type counterpart. Thus, this leads to an immunological ignorance of the B16 melanoma by CD4+ T cells from the MARCH1-deficient mice.

CHAPITRE 5: CONCLUSION

We aimed to elucidate the role of MARCH1 in the development of B16 melanoma. Our findings demonstrate that MARCH1 deficiency in mice largely impacts this experimental model of melanoma. Indeed, we found that MARCH1 KO mice developed more aggressive and larger tumors than the wild-type mice. Furthermore, we pursued the *in vitro* studies of Ishido and his colleagues on the activation of naïve CD4+ T cells by MARCH1 KO DCs, with this *in vivo* mouse model. We found that the aggressive development of B16 tumors in the MARCH1 KO mice is apparently due to the impaired priming of naïve CD4+ T cells in the lymph nodes by DCs. Therefore, a significantly reduced infiltration of primed CD4+ T cells to the tumor is observed in the MARCH1 KO mice. As these cells are required for an efficient anti-tumor response, this leads to a rapid and aggressive tumor growth in the MARCH1 KO mice. Future work is required, for example the use of a MHC class II knock-in mice cohort, to further highlight the specific impact of MARCH1 deficiency. Moreover, the CD4+ T cell response can be assessed further by MHC class II tetramers carrying OVA peptide in a B16-OVA model. Finally, as the MHC class II-mediated antigen presentation pathway is essential for the mounting of efficient anti-tumor responses, methods that will improve antigen presentation during tumor immunosurveillance will be critical for the development of the new generation of immune targets for cancer immunotherapy.

CHAPTER 6: BIBLIOGRAPHY

1. Kasahara M. The chromosomal duplication model of the major histocompatibility complex. Immunol Rev. 1999;167:17-32.
2. Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. Annu Rev Immunol. 2013;31:443-73.
3. Groettrup M, Kraft R, Kostka S, Standera S, Stohwasser R, Kloetzel PM. A third interferon-gamma-induced subunit exchange in the 20S proteasome. Eur J Immunol. 1996;26(4):863-9.
4. Hayashi M, Ishibashi T, Tanaka K, Kasahara M. The mouse genes encoding the third pair of beta-type proteasome subunits regulated reciprocally by IFN-gamma: structural comparison, chromosomal localization, and analysis of the promoter. J Immunol. 1997;159(6):2760-70.
5. Maupin-Furlow J. Proteasomes and protein conjugation across domains of life. Nat Rev Microbiol. 2012;10(2):100-11.
6. Huotari J, Helenius A. Endosome maturation. EMBO J. 2011;30(17):3481-500.
7. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature. 2003;421(6925):852-6.
8. Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. Science. 2003;300(5617):337-9.
9. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. Science. 2003;300(5617):339-42.
10. Mach B, Steimle V, Reith W. MHC class II-deficient combined immunodeficiency: a disease of gene regulation. Immunol Rev. 1994;138:207-21.
11. Ting JP, Trowsdale J. Genetic control of MHC class II expression. Cell. 2002;109 Suppl:S21-33.
12. Harton JA, Ting JP. Class II transactivator: mastering the art of major histocompatibility complex expression. Mol Cell Biol. 2000;20(17):6185-94.
13. Landmann S, Muhlethaler-Mottet A, Bernasconi L, Suter T, Waldburger JM, Masternak K, et al. Maturation of dendritic cells is accompanied by rapid transcriptional silencing of class II transactivator (CIITA) expression. J Exp Med. 2001;194(4):379-91.
14. Wright KL, Ting JP. Epigenetic regulation of MHC-II and CIITA genes. Trends Immunol. 2006;27(9):405-12.
15. Shin JS, Ebersold M, Pypaert M, Delamarre L, Hartley A, Mellman I. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. Nature. 2006;444(7115):115-8.
16. van Niel G, Wubbolts R, Ten Broeke T, Buschow SI, Ossendorp FA, Melief CJ, et al. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. Immunity. 2006;25(6):885-94.

17. Haglund K, Dikic I. Ubiquitylation and cell signaling. EMBO J. 2005;24(19):3353-9.
18. Mukhopadhyay D, Riezman H. Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science. 2007;315(5809):201-5.
19. Pickart CM. Mechanisms underlying ubiquitination. Annu Rev Biochem. 2001;70:503-33.
20. Behrends C, Harper JW. Constructing and decoding unconventional ubiquitin chains. Nat Struct Mol Biol. 2011;18(5):520-8.
21. Callis J. The ubiquitination machinery of the ubiquitin system. Arabidopsis Book. 2014;12:e0174.
22. Goto E, Ishido S, Sato Y, Ohgimoto S, Ohgimoto K, Nagano-Fujii M, et al. c-MIR, a human E3 ubiquitin ligase, is a functional homolog of herpesvirus proteins MIR1 and MIR2 and has similar activity. J Biol Chem. 2003;278(17):14657-68.
23. Boname JM, Lehner PJ. What has the study of the K3 and K5 viral ubiquitin E3 ligases taught us about ubiquitin-mediated receptor regulation? Viruses. 2011;3(2):118-31.
24. Coscoy L, Ganem D. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. Proc Natl Acad Sci U S A. 2000;97(14):8051-6.
25. Haque M, Ueda K, Nakano K, Hirata Y, Parravicini C, Corbellino M, et al. Major histocompatibility complex class I molecules are down-regulated at the cell surface by the K5 protein encoded by Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8. J Gen Virol. 2001;82(Pt 5):1175-80.
26. Bartee E, Mansouri M, Hovey Nerenberg BT, Gouveia K, Fruh K. Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. J Virol. 2004;78(3):1109-20.
27. Matsuki Y, Ohmura-Hoshino M, Goto E, Aoki M, Mito-Yoshida M, Uematsu M, et al. Novel regulation of MHC class II function in B cells. EMBO J. 2007;26(3):846-54.
28. De Gassart A, Camosseto V, Thibodeau J, Ceppi M, Catalan N, Pierre P, et al. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proc Natl Acad Sci U S A. 2008;105(9):3491-6.
29. Ohmura-Hoshino M, Matsuki Y, Mito-Yoshida M, Goto E, Aoki-Kawasumi M, Nakayama M, et al. Cutting edge: requirement of MARCH-I-mediated MHC II ubiquitination for the maintenance of conventional dendritic cells. J Immunol. 2009;183(11):6893-7.
30. Oh J, Wu N, Baravalle G, Cohn B, Ma J, Lo B, et al. MARCH1-mediated MHCII ubiquitination promotes dendritic cell selection of natural regulatory T cells. J Exp Med. 2013;210(6):1069-77.
31. Laufer TM, DeKoning J, Markowitz JS, Lo D, Glimcher LH. Unopposed positive

selection and autoreactivity in mice expressing class II MHC only on thymic cortex. Nature. 1996;383(6595):81-5.
32. Hsieh CS, Lee HM, Lio CW. Selection of regulatory T cells in the thymus. Nat Rev Immunol. 2012;12(3):157-67.
33. Liu H, Jain R, Guan J, Vuong V, Ishido S, La Gruta NL, et al. Ubiquitin ligase MARCH 8 cooperates with CD83 to control surface MHC II expression in thymic epithelium and CD4 T cell selection. J Exp Med. 2016.
34. von Rohrscheidt J, Petrozziello E, Nedjic J, Federle C, Krzyzak L, Ploegh HL, et al. Thymic CD4 T cell selection requires attenuation of March8-mediated MHCII turnover in cortical epithelial cells through CD83. J Exp Med. 2016.
35. Tze LE, Horikawa K, Domaschenz H, Howard DR, Roots CM, Rigby RJ, et al. CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. J Exp Med. 2011;208(1):149-65.
36. Thibodeau J, Bourgeois-Daigneault MC, Lapointe R. Targeting the MHC Class II antigen presentation pathway in cancer immunotherapy. Oncoimmunology. 2012;1(6):908-16.
37. Jabbour M, Campbell EM, Fares H, Lybarger L. Discrete domains of MARCH1 mediate its localization, functional interactions, and posttranscriptional control of expression. J Immunol. 2009;183(10):6500-12.
38. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986;136(7):2348-57.
39. Fernandez-Botran R, Sanders VM, Mosmann TR, Vitetta ES. Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. J Exp Med. 1988;168(2):543-58.
40. Koppelman B, Neefjes JJ, de Vries JE, de Waal Malefyt R. Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. Immunity. 1997;7(6):861-71.
41. Thibodeau J, Bourgeois-Daigneault MC, Huppe G, Tremblay J, Aumont A, Houde M, et al. Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. Eur J Immunol. 2008;38(5):1225-30.
42. Gazzinelli RT, Wysocka M, Hieny S, Scharton-Kersten T, Cheever A, Kuhn R, et al. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol. 1996;157(2):798-805.
43. Sabat R. IL-10 family of cytokines. Cytokine Growth Factor Rev. 2010;21(5):315-24.
44. Chadban SJ, Tesch GH, Foti R, Lan HY, Atkins RC, Nikolic-Paterson DJ. Interleukin-10 differentially modulates MHC class II expression by mesangial cells and macrophages in vitro and in vivo. Immunology. 1998;94(1):72-8.

45. Trinchieri G. Interleukin-10 production by effector T cells: Th1 cells show self control. J Exp Med. 2007;204(2):239-43.
46. Demangel C, Bertolino P, Britton WJ. Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. Eur J Immunol. 2002;32(4):994-1002.
47. Itoh K, Hirohata S. The role of IL-10 in human B cell activation, proliferation, and differentiation. J Immunol. 1995;154(9):4341-50.
48. Mocellin S, Panelli MC, Wang E, Nagorsen D, Marincola FM. The dual role of IL-10. Trends Immunol. 2003;24(1):36-43.
49. Mocellin S, Marincola F, Rossi CR, Nitti D, Lise M. The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. Cytokine Growth Factor Rev. 2004;15(1):61-76.
50. Vicari AP, Trinchieri G. Interleukin-10 in viral diseases and cancer: exiting the labyrinth? Immunol Rev. 2004;202:223-36.
51. Mocellin S, Marincola FM, Young HA. Interleukin-10 and the immune response against cancer: a counterpoint. J Leukoc Biol. 2005;78(5):1043-51.
52. Mumm JB, Oft M. Pegylated IL-10 induces cancer immunity: the surprising role of IL-10 as a potent inducer of IFN-gamma-mediated CD8(+) T cell cytotoxicity. Bioessays. 2013;35(7):623-31.
53. Wang L, Liu JQ, Talebian F, Liu Z, Yu L, Bai XF. IL-10 enhances CTL-mediated tumor rejection by inhibiting highly suppressive CD4+ T cells and promoting CTL persistence in a murine model of plasmacytoma. Oncoimmunology. 2015;4(7):e1014232.
54. Canadian Cancer Society's Advisory Committee on Cancer Statistics. Canadian Cancer Statistics 2015. In: Canada PHAo, editor. Toronto, ON: Canadian Cancer Society; 2015.
55. Canadian Cancer Society. What is melanoma? 2016 [Available from: http://www.cancer.ca/en/cancer-information/cancer-type/skin-melanoma/melanoma/?region=on.
56. Soong SJ, Harrison RA, McCarthy WH, Urist MM, Balch CM. Factors affecting survival following local, regional, or distant recurrence from localized melanoma. J Surg Oncol. 1998;67(4):228-33.
57. Tas F. Metastatic behavior in melanoma: timing, pattern, survival, and influencing factors. J Oncol. 2012;2012:647684.
58. Maddodi N, Setaluri V. Role of UV in cutaneous melanoma. Photochem Photobiol. 2008;84(2):528-36.
59. World Health Organization. Climate change and human health : risks and responses. Summary. Geneva, Switzerland2003.
60. Bhatia S, Tykodi SS, Thompson JA. Treatment of metastatic melanoma: an overview. Oncology (Williston Park). 2009;23(6):488-96.
61. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of

the BRAF gene in human cancer. Nature. 2002;417(6892):949-54.
62. Peyssonnaux C, Eychene A. The Raf/MEK/ERK pathway: new concepts of activation. Biol Cell. 2001;93(1-2):53-62.
63. Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. Nature. 2010;468(7326):973-7.
64. Sullivan RJ, Flaherty KT. Resistance to BRAF-targeted therapy in melanoma. Eur J Cancer. 2013;49(6):1297-304.
65. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363(8):711-23.
66. Barbee MS, Ogunniyi A, Horvat TZ, Dang TO. Current status and future directions of the immune checkpoint inhibitors ipilimumab, pembrolizumab, and nivolumab in oncology. Ann Pharmacother. 2015;49(8):907-37.
67. Mahoney KM, Freeman GJ, McDermott DF. The Next Immune-Checkpoint Inhibitors: PD-1/PD-L1 Blockade in Melanoma. Clin Ther. 2015;37(4):764-82.
68. Lin JY, Fisher DE. Melanocyte biology and skin pigmentation. Nature. 2007;445(7130):843-50.
69. Cichorek M, Wachulska M, Stasiewicz A, Tyminska A. Skin melanocytes: biology and development. Postepy Dermatol Alergol. 2013;30(1):30-41.
70. Hirobe T. Structure and function of melanocytes: microscopic morphology and cell biology of mouse melanocytes in the epidermis and hair follicle. Histol Histopathol. 1995;10(1):223-37.
71. Brenner M, Hearing VJ. The protective role of melanin against UV damage in human skin. Photochem Photobiol. 2008;84(3):539-49.
72. Jimbow K. Current update and trends in melanin pigmentation and melanin biology. Keio J Med. 1995;44(1):9-18.
73. Wu X, Hammer JA, 3rd. Making sense of melanosome dynamics in mouse melanocytes. Pigment Cell Res. 2000;13(4):241-7.
74. Sulaimon SS, Kitchell BE. The biology of melanocytes. Vet Dermatol. 2003;14(2):57-65.
75. Gates RR, Zimmermann AA. Comparison of skin color with melanin content. J Invest Dermatol. 1953;21(6):339-48.
76. Byers HR, Maheshwary S, Amodeo DM, Dykstra SG. Role of cytoplasmic dynein in perinuclear aggregation of phagocytosed melanosomes and supranuclear melanin cap formation in human keratinocytes. J Invest Dermatol. 2003;121(4):813-20.
77. Sinha RP, Hader DP. UV-induced DNA damage and repair: a review. Photochem Photobiol Sci. 2002;1(4):225-36.
78. Kolgen W, Both H, van Weelden H, Guikers KL, Bruijnzeel-Koomen CA, Knol EF, et al. Epidermal langerhans cell depletion after artificial ultraviolet B irradiation of

human skin in vivo: apoptosis versus migration. J Invest Dermatol. 2002;118(5):812-7.
79. Nithiuthai S, Allen JR. Effects of ultraviolet irradiation on the acquisition and expression of tick resistance in guinea-pigs. Immunology. 1984;51(1):153-9.
80. Aberer W, Stingl G, Stingl-Gazze LA, Wolff K. Langerhans cells as stimulator cells in the murine primary epidermal cell-lymphocyte reaction: alteration by UV-B irradiation. J Invest Dermatol. 1982;79(2):129-35.
81. Kollias N, Sayre RM, Zeise L, Chedekel MR. Photoprotection by melanin. J Photochem Photobiol B. 1991;9(2):135-60.
82. Zaidi MR, Day CP, Merlino G. From UVs to metastases: modeling melanoma initiation and progression in the mouse. J Invest Dermatol. 2008;128(10):2381-91.
83. Smalley KS, Brafford PA, Herlyn M. Selective evolutionary pressure from the tissue microenvironment drives tumor progression. Semin Cancer Biol. 2005;15(6):451-9.
84. Haass NK, Smalley KS, Li L, Herlyn M. Adhesion, migration and communication in melanocytes and melanoma. Pigment Cell Res. 2005;18(3):150-9.
85. Marrero B, Messina JL, Heller R. Generation of a tumor spheroid in a microgravity environment as a 3D model of melanoma. In Vitro Cell Dev Biol Anim. 2009;45(9):523-34.
86. Schoop VM, Mirancea N, Fusenig NE. Epidermal organization and differentiation of HaCaT keratinocytes in organotypic coculture with human dermal fibroblasts. J Invest Dermatol. 1999;112(3):343-53.
87. Hutmacher DW, Loessner D, Rizzi S, Kaplan DL, Mooney DJ, Clements JA. Can tissue engineering concepts advance tumor biology research? Trends Biotechnol. 2010;28(3):125-33.
88. Walker GJ, Soyer HP, Terzian T, Box NF. Modelling melanoma in mice. Pigment Cell Melanoma Res. 2011;24(6):1158-76.
89. Gallagher CH, Canfield PJ, Greenoak GE, Reeve VE. Characterization and histogenesis of tumors in the hairless mouse produced by low-dosage incremental ultraviolet radiation. J Invest Dermatol. 1984;83(3):169-74.
90. Walker GJ, Hayward NK. Pathways to melanoma development: lessons from the mouse. J Invest Dermatol. 2002;119(4):783-92.
91. McKinney AJ, Holmen SL. Animal models of melanoma: a somatic cell gene delivery mouse model allows rapid evaluation of genes implicated in human melanoma. Chin J Cancer. 2011;30(3):153-62.
92. Chin L, Garraway LA, Fisher DE. Malignant melanoma: genetics and therapeutics in the genomic era. Genes Dev. 2006;20(16):2149-82.
93. Kuzu OF, Nguyen FD, Noory MA, Sharma A. Current State of Animal (Mouse) Modeling in Melanoma Research. Cancer Growth Metastasis. 2015;8(Suppl 1):81-94.
94. Kelland LR. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. Eur J Cancer. 2004;40(6):827-36.

95. Merlino G, Flaherty K, Acquavella N, Day CP, Aplin A, Holmen S, et al. Meeting report: The future of preclinical mouse models in melanoma treatment is now. Pigment Cell Melanoma Res. 2013;26(4):E8-E14.
96. Tentler JJ, Tan AC, Weekes CD, Jimeno A, Leong S, Pitts TM, et al. Patient-derived tumour xenografts as models for oncology drug development. Nat Rev Clin Oncol. 2012;9(6):338-50.
97. Pickup MW, Mouw JK, Weaver VM. The extracellular matrix modulates the hallmarks of cancer. EMBO Rep. 2014;15(12):1243-53.
98. Teicher BA. Tumor Models in Cancer Research. New York: Heidelberg and London; 2011 2010.
99. Klarquist JS, Janssen EM. Melanoma-infiltrating dendritic cells: Limitations and opportunities of mouse models. Oncoimmunology. 2012;1(9):1584-93.
100. Fidler IJ. Biological behavior of malignant melanoma cells correlated to their survival in vivo. Cancer Res. 1975;35(1):218-24.
101. Bosmann HB, Bieber GF, Brown AE, Case KR, Gersten DM, Kimmerer TW, et al. Biochemical parameters correlated with tumour cell implantation. Nature. 1973;246(5434):487-9.
102. Overwijk WW, Restifo NP. B16 as a mouse model for human melanoma. Curr Protoc Immunol. 2001;Chapter 20:Unit 20 1.
103. Bohm W, Thoma S, Leithauser F, Moller P, Schirmbeck R, Reimann J. T cell-mediated, IFN-gamma-facilitated rejection of murine B16 melanomas. J Immunol. 1998;161(2):897-908.
104. Celluzzi CM, Mayordomo JI, Storkus WJ, Lotze MT, Falo LD, Jr. Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. J Exp Med. 1996;183(1):283-7.
105. Qin Z, Blankenstein T. CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. Immunity. 2000;12(6):677-86.
106. Antony PA, Piccirillo CA, Akpinarli A, Finkelstein SE, Speiss PJ, Surman DR, et al. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. J Immunol. 2005;174(5):2591-601.
107. Cabrera T, Ruiz-Cabello F, Garrido F. Biological implications of HLA-DR expression in tumours. Scand J Immunol. 1995;41(4):398-406.
108. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H. The central role of CD4(+) T cells in the antitumor immune response. J Exp Med. 1998;188(12):2357-68.
109. Mumberg D, Monach PA, Wanderling S, Philip M, Toledano AY, Schreiber RD, et al. CD4(+) T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-gamma. Proc Natl Acad Sci U S A. 1999;96(15):8633-8.

110. Perez-Diez A, Joncker NT, Choi K, Chan WF, Anderson CC, Lantz O, et al. CD4 cells can be more efficient at tumor rejection than CD8 cells. Blood. 2007;109(12):5346-54.
111. Corthay A, Skovseth DK, Lundin KU, Rosjo E, Omholt H, Hofgaard PO, et al. Primary antitumor immune response mediated by CD4+ T cells. Immunity. 2005;22(3):371-83.
112. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol. 1993;11:571-611.
113. Fleischmann CM, Fleischmann WR, Jr. Enhanced in vitro macrophage cytotoxicity against interferon-treated B16 melanoma cells. J Biol Regul Homeost Agents. 1995;9(4):139-45.
114. Fleischmann CM, Stanton GJ, Fleischmann WR, Jr. Enhanced in vivo sensitivity of in vitro interferon-treated B16 melanoma cells to CD8 cells and activated macrophages. J Interferon Cytokine Res. 1996;16(10):805-12.
115. Ishikawa R, Kajikawa M, Ishido S. Loss of MHC II ubiquitination inhibits the activation and differentiation of CD4 T cells. Int Immunol. 2014;26(5):283-9.
116. Holmdahl R, Malissen B. The need for littermate controls. Eur J Immunol. 2012;42(1):45-7.
117. Yokota J. Tumor progression and metastasis. Carcinogenesis. 2000;21(3):497-503.
118. Folkman J, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature. 1989;339(6219):58-61.
119. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996;86(3):353-64.
120. Hadrup S, Donia M, Thor Straten P. Effector CD4 and CD8 T cells and their role in the tumor microenvironment. Cancer Microenviron. 2013;6(2):123-33.
121. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology. 2007;121(1):1-14.
122. Gimotty PA, Van Belle P, Elder DE, Murry T, Montone KT, Xu X, et al. Biologic and prognostic significance of dermal Ki67 expression, mitoses, and tumorigenicity in thin invasive cutaneous melanoma. J Clin Oncol. 2005;23(31):8048-56.
123. van Houdt IS, Sluijter BJ, Moesbergen LM, Vos WM, de Gruijl TD, Molenkamp BG, et al. Favorable outcome in clinically stage II melanoma patients is associated with the presence of activated tumor infiltrating T-lymphocytes and preserved MHC class I antigen expression. Int J Cancer. 2008;123(3):609-15.
124. Gooden MJ, de Bock GH, Leffers N, Daemen T, Nijman HW. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer. 2011;105(1):93-103.
125. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. Nat Rev Immunol. 2005;5(8):617-28.
126. Varney ML, Singh S, Backora M, Chen Z, Singh RK. Lymphangiogenesis and anti-

tumor immune responses. Curr Mol Med. 2009;9(6):694-701.
127. Ginhoux F, Liu K, Helft J, Bogunovic M, Greter M, Hashimoto D, et al. The origin and development of nonlymphoid tissue CD103+ DCs. J Exp Med. 2009;206(13):3115-30.
128. Shortman K, Heath WR. The CD8+ dendritic cell subset. Immunol Rev. 2010;234(1):18-31.
129. Villadangos JA, Young L. Antigen-presentation properties of plasmacytoid dendritic cells. Immunity. 2008;29(3):352-61.
130. Preynat-Seauve O, Schuler P, Contassot E, Beermann F, Huard B, French LE. Tumor-infiltrating dendritic cells are potent antigen-presenting cells able to activate T cells and mediate tumor rejection. J Immunol. 2006;176(1):61-7.
131. Romani N, Clausen BE, Stoitzner P. Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin. Immunol Rev. 2010;234(1):120-41.
132. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol. 2013;31:563-604.
133. Henri S, Guilliams M, Poulin LF, Tamoutounour S, Ardouin L, Dalod M, et al. Disentangling the complexity of the skin dendritic cell network. Immunol Cell Biol. 2010;88(4):366-75.
134. Villadangos JA, Schnorrer P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol. 2007;7(7):543-55.
135. Thompson ED, Enriquez HL, Fu YX, Engelhard VH. Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. J Exp Med. 2010;207(8):1791-804.
136. Wang HY, Fu T, Wang G, Zeng G, Perry-Lalley DM, Yang JC, et al. Induction of CD4(+) T cell-dependent antitumor immunity by TAT-mediated tumor antigen delivery into dendritic cells. J Clin Invest. 2002;109(11):1463-70.
137. Goldszmid RS, Idoyaga J, Bravo AI, Steinman R, Mordoh J, Wainstok R. Dendritic cells charged with apoptotic tumor cells induce long-lived protective CD4+ and CD8+ T cell immunity against B16 melanoma. J Immunol. 2003;171(11):5940-7.
138. Hahne M, Rimoldi D, Schroter M, Romero P, Schreier M, French LE, et al. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. Science. 1996;274(5291):1363-6.
139. Aspord C, Leccia MT, Charles J, Plumas J. Melanoma hijacks plasmacytoid dendritic cells to promote its own progression. Oncoimmunology. 2014;3(1):e27402.
140. Di Domizio J, Demaria O, Gilliet M. Plasmacytoid dendritic cells in melanoma: can we revert bad into good? J Invest Dermatol. 2014;134(7):1797-800.
141. Liu C, Lou Y, Lizee G, Qin H, Liu S, Rabinovich B, et al. Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice. J Clin Invest. 2008;118(3):1165-75.

142. Gerner MY, Mescher MF. Antigen processing and MHC-II presentation by dermal and tumor-infiltrating dendritic cells. J Immunol. 2009;182(5):2726-37.
143. Wolf D, Sopper S, Pircher A, Gastl G, Wolf AM. Treg(s) in Cancer: Friends or Foe? J Cell Physiol. 2015;230(11):2598-605.
144. Quezada SA, Peggs KS, Simpson TR, Shen Y, Littman DR, Allison JP. Limited tumor infiltration by activated T effector cells restricts the therapeutic activity of regulatory T cell depletion against established melanoma. J Exp Med. 2008;205(9):2125-38.
145. Klages K, Mayer CT, Lahl K, Loddenkemper C, Teng MW, Ngiow SF, et al. Selective depletion of Foxp3+ regulatory T cells improves effective therapeutic vaccination against established melanoma. Cancer Res. 2010;70(20):7788-99.
146. Imai H, Saio M, Nonaka K, Suwa T, Umemura N, Ouyang GF, et al. Depletion of CD4+CD25+ regulatory T cells enhances interleukin-2-induced antitumor immunity in a mouse model of colon adenocarcinoma. Cancer Sci. 2007;98(3):416-23.
147. Bouard D, Alazard-Dany D, Cosset FL. Viral vectors: from virology to transgene expression. Br J Pharmacol. 2009;157(2):153-65.
148. Swift S, Lorens J, Achacoso P, Nolan GP. Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. Curr Protoc Immunol. 2001;Chapter 10:Unit 10 7C.