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# CIITA-induced MHC class II Expression in Tumor Cells: A Novel Universal Strategy of Anti-Tumor Vaccination

**Ph.D. Thesis of**

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*Dedicated to:*

*My mother, my father and my two brothers Shadi and Houssam… only your support made everything possible.*

*My little cousins Vanessa and Sara … follow your dreams, that's the only right path.*

*Dr. Giovanna TOSI who taught me a lot about science, dedication, strength, patience, hope and most importantly that life is not about winning but how we fight till the end... knowing you was a privilege… may your soul rest in peace.*

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# Table of Contents

**Abstract** ..... 6

**Introduction** ..... 7

**Part I.** ..... 9

**Chapter 1: Overview on the immune system** ..... 9

***1. The innate immune response*** ..... 9

***2. The adaptive immune response*** ..... 9

2.1- Humoral immune response ..... 10

2.2- Cellular immune response ..... 11

*2.2-1. CD8+ T cells* ..... 12

*2.2-2. CD4+ T cells* ..... 14

**Chapter 2: Major Histocompatibility System and Antigen Presentation** ..... 17

***1. MHC-I Molecules*** ..... 18

***2. MHC-II Molecules*** ..... 20

2.1- MHC-II molecules and CIITA ..... 22

**Chapter 3: Cancer Immunsurveillance, Escape and Immunotherapy** ..... 24

**Part II.** ..... 26

**II.1- Our Approach and previous results** ..... 26

**II.2- Aim of the Project** ..... 27

**Part III.** ..... 28

**Chapter 1: Materials and Methods** ..... 28

***1. Transfection and phenotypic analysis of tumor models*** ..... 28

***2. In vitro Proliferation rate assay*** ..... 28

***3. Vaccination and challenge*** ..... 28

***4. Ex vivo MC38-CIITA tumors analysis*** ..... 29

***5. ELISA analysis*** ..... 29

***6. Adoptive Cell Transfer*** ..... 29

***7. Dendritic Cells depletion in CD11c.DTR transgenic C57BL/6 mice*** ..... 30

***8. Macrophages depletion with liposomal Clodronate*** ..... 31

***9. Statistical analysis*** ..... 31

**Chapter 2: Results** ..... 32

***1. CIITA-transfected MHC-II positive MC38 clone generation*** ..... 32

*2. CIITA-driven MHC-II+ tumor cells are rejected or strongly retarded in their growth* . 34
*3. MC38-CIITA tumor cells trigger a long lasting adaptive immune response* ................. 35
*4. Loss of MHC-I and MHC-II by the MC38-CIITA growing tumors* ............................... 36
*5. High IFN-γ secretion by splenocytes of immune mice* .................................................... 37
*6. The long lasting immune response triggered by MC38-CIITA is orchestrated by CD4+ T cells* ........................................................................................................................ 38
*7. CIITA driven MHC-II positive MC38 tumor cells act as surrogate APCs for priming and activating CD4+ TH cells* ........................................................................................ 39
**Chapter 3: Discussion and Conclusion** ................................................................... 44
**Abbreviations List** ................................................................................................ 47
**Schemes and Figures Lists** ................................................................................... 49
**Publications** .......................................................................................................... 51
**References** ............................................................................................................. 52

# Abstract

Priming and activation of CD4+ T helper (TH) cells against tumor associated antigens can be achieved after their recognition on antigen presenting cells (APCs) only within the context of MHC class II (MHC-II) molecules. We previously reported successful triggering of TH-specific long lasting anti-tumor immune response in H-$2^d$ haplotype BALB/c mice using tumor cells genetically modified to express endogenous MHC-II genes (I-A and I-E) after transfection with CIITA (MHC-II transactivator). Now, we aim to investigate the pertinence of this approach in H-$2^b$ haplotype C57BL/6 mice that only express I-A molecules due to a defect in their I-Eα gene. MC38 colon carcinoma cells of the H-$2^b$ haplotype were stably transfected with CIITA. Selected MHC-II positive clones were injected into C57BL/6 mice. Complete rejection or significant growth retardation as compared to MHC-II negative parental tumor was obtained. Subsequent challenge of the protected mice with parental tumor proved that the CIITA-transfected tumor engendered efficient anti-tumor vaccination. Then, adoptive cell transfer from the vaccinated mice to naïve recipients demonstrated that CD4+ TH cells orchestrate the anti-tumor protection. Finally, the use of CD11c.DTR transgenic mice in which conditional depletion of dendritic cells (DCs) can be performed, and the use of Liposomal Clodronate as a depletion agent for macrophages, proved that CIITA-driven MHC-II positive tumor cells act as surrogate APCs for priming and activating CD4+ TH cells, without the need of either DCs or macrophages.

These results demonstrate the validity of CIITA-driven MHC-II+ tumor cells as anti-tumor vaccination tool in mouse models of different haplotypes. Moreover, they prove that expression of a single MHC-II restriction element in tumor cells is sufficient to trigger anti-tumor CD4+ TH cells and, more importantly, demonstrate for the first time that CIITA-driven MHC-II expressing tumor cells can act as professional APCs *in vivo* to prime naïve tumor-specific CD4+ TH cells.

# Introduction

The rise of the global cancer burden to 4.1 million new cases per year, 8.2 million cancer-caused deaths in 2012 and the expectations that the number of new cases will increase by about 70% over the next two decades strongly urge additional basic and applied research efforts to counteract this plague [1]. Nowadays, a significant part of cancer research is directed towards immunotherapy due to the evident existence of an immune response against neoplastic cells and the fact that cancer does not take off in an organism unless cancer cells display reduced immunogenicity or exhibit an increased inhibiting capacity against protective anti-tumor immune responses [2], [3].

It is widely accepted that tumor-specific CD4+ TH cells play a pivotal role in anti-cancer immunity, mainly because they provide help in proliferation, differentiation and maintenance of CD8+ T cells that are believed to be the major anti-tumor effector cells. In fact, priming of CD8+ T cells in the absence of CD4+ TH cells lead to their “helpless” activation in terms of clonal expansion and acquisition of cytolytic function. Nevertheless, most of the strategies of active and passive cancer immunotherapies have focused on stimulating primarily cytolytic CD8+ T cells (CTL) with tumor-associated antigens (TAA) presented by MHC-I molecules, which are expressed on the surface of the majority of tumor cells and thus are more easily identifiable with respect to MHC-II restricted TAA by biochemical tools [4], [5], [6], [7], [8].

In a new approach aiming instead at the optimal stimulation of tumor-specific TH cells, our group already launched and published many studies based on animal vaccination using tumor cells transduced with the AIR-1-encoded CIITA, the MHC-II transactivator [9]. The idea underlying this approach was to make the tumor cells expressing MHC-II molecules, hoping that they would present MHC-II-restricted TAA directly to the TH cells and thus act as surrogate APCs of their own tumor antigens *in vivo.* Using this approach, we proved that a complete tumor rejection and specific antitumor memory against tumors of different histotypes can be achieved in the Balb/c mouse model of the H-$2^d$ haplotype [10], [11], [12].

In the present work we assessed the pertinence of this vaccination approach in tumor models of the C57BL/6 mouse expressing the H-$2^b$ haplotype. C57BL/6 mice hold a resilient challenge because one of the two possible MHC class II molecules, I-A and I-E, namely the I-E molecule

cannot be expressed because of a mutation in its I-Eα gene. Thus only the I-A molecule is expressed on the surface of the H-$2^b$ cells [13], [14].

Moreover, a transgenic C57BL/6 mouse model is available in which it is possible to transiently deplete DCs believed to be the crucial APCs for priming CD4+ TH cells [15] offering the possibility to unambiguously assess whether CIITA-driven MHC-II-expressing tumor cells could still trigger a protective anti-tumor response *in vivo* in absence of the major source of professional APCs.

# Part I.
# Chapter 1: Overview on The Immune System

The immune system is an interactive network of lymphoid organs, cells, humoral factors, and cytokines. The essential function of this system is to protect the organism against all kinds of disease-causing microorganisms or pathogens like viruses, bacteria, fungi, parasites. The reaction of the immune system against pathogens is known as the immune response and depending on its speed and specificity can be divided into two arms: The innate and adaptive immune responses [16], [17].

## *1. The innate immune response*

Innate immunity (also called natural or native immunity) provides the early line of defense against pathogens. It is a highly conserved form of response and is seen in even the simplest animals confirming its importance in survival. It consists of cellular and biochemical defense mechanisms that are in place even before infection and react rapidly, in terms of hours, to the infections. These mechanisms are not specific for each pathogen and respond in essentially the same way to repeated exposures of microbes without distinguishing the differences between them. The principal components of innate immunity are (1) physical and chemical barriers, such as epithelia and antimicrobial chemicals produced at epithelial surfaces; (2) phagocytic cells (neutrophils, macrophages), dendritic cells, and natural killer cells and (3) blood proteins, including members of the complement system and other mediators of inflammation [17], [18].

## *2. The adaptive immune response*

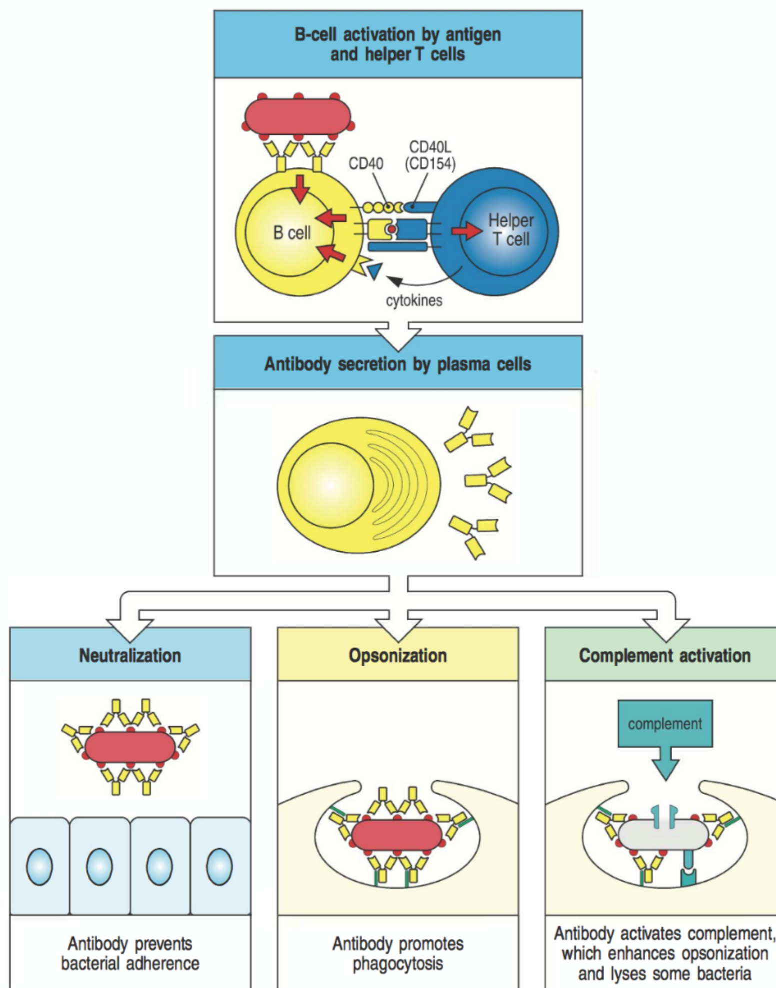
Adaptive immunity is triggered when an infection overwhelms the innate defense mechanisms and generates a threshold level of antigen. It is highly specific to a particular pathogen and can also provide long-lasting protection called immunological memory. The adaptive immune response is initiated in the peripheral lymphoid organs like lymph nodes, spleen, and the mucosa-associated lymphoid tissues such as the Peyer's patches of the gut. The main components of the adaptive immune response are the two primary types of lymphocytes, B and T. They both originate from stem cells in the bone marrow then some of them migrate to the thymus, where they mature into T cells while others remain in the bone marrow, where they develop into B cells.

Two distinct sets of highly variable receptor molecules serve as antigen-specific receptors on B cells (BCR) and T cells (TCR), and the adaptive immune response can be divided into two types depending on which one of these two lymphocyte subpopulations, B or T, will be responsible of the response [16].

### *2.1- Humoral immune response*

Through their BCR, the B cells bind native proteins, glycoproteins, and polysaccharides, as well as whole virus particles and bacterial cells, by recognizing epitopes on their surfaces. Then the B-cell antigen receptor delivers the bound antigen to intracellular sites, where it can be degraded to give peptides that are returned to the B-cell surface bound to major histocompatibility complexes class II (MHC-II) molecules. These peptide:MHC-II complexes, are recognized by antigen-specific TH cells that have already differentiated in response to the same pathogen. The B cell-T cell interaction is strengthen by the binding of co-stimulatory molecules, CD40-CD40 ligand (CD40L) respectively, that start to be expressed on the surface of these cells after the antigen recognition. As a result of this interaction, the effector T cells secrete cytokines that cause the B cell to proliferate and its progeny to differentiate into antibody-secreting cells or plasma cells and into memory B cells (Scheme 1, top first and second panels) [16].

Antibodies, that are the characteristic targeted effectors of the humoral immune response, protect the host from infection in three main ways. First, they can inhibit the toxic effects of pathogens by binding to them, which is termed neutralization (Scheme 1, bottom left panel). Second, by coating the pathogens they can enable accessory cells that recognize the constant fragments (Fc) portions of antibodies to ingest and kill the pathogen, a process called opsonization (Scheme 1, bottom center panel). Third, antibodies can trigger the activation of the complement system. Complement proteins strongly enhance opsonization, and can directly kill certain bacterial cells (Scheme 1, bottom right panel) [16] .




**Scheme 1** The main steps of the humoral immune response (from Janeway Immunobiology, 8th edition)

## *2.2- Cellular immune response*

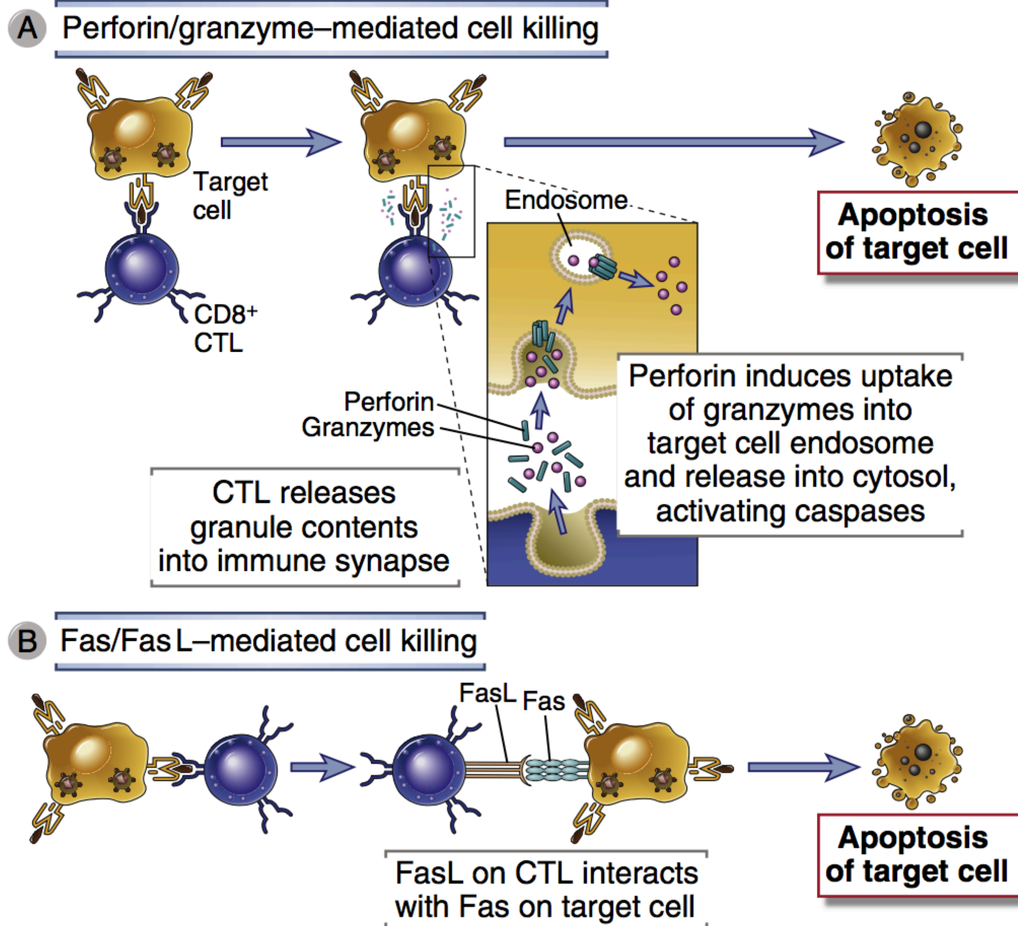
The cellular immune response is also called T cell-mediated immune response since the T lymphocytes are its main effectors. Unlike B cells, T cells only recognize antigens that are displayed on cells surface. In order to trigger a T cell-mediated immune response, a naïve T cell must meet its specific antigen, presented as a peptide within the context of MHC molecules on the surface of an APC and thus be induced to proliferate and differentiate into antigen-specific effector cells. The peptides presented by MHC molecules can be derived for example from pathogens that replicate within cells, such as viruses or intracellular bacteria, or from pathogens or their products that cells have taken up from the extracellular fluid. Unstimulated naïve T cells fall into two large classes, one that carries the co-receptor CD8 on its surface and the other the co-receptor CD4. Upon antigen recognition, naïve T cells differentiate into several functional classes of effector T cells that are specialized for different activities. Naïve CD8+ T cells

recognize pathogen peptides presented by MHC-I molecules on the surface of APCs and differentiate into cytotoxic effector T cells that recognize and kill all infected cells. CD4+ T cells have a more flexible repertoire of effector activities. After recognition of pathogen peptides presented by MHC-II molecules on the surface of APCs, naïve CD4+ T cells can differentiate in distinct pathways to generate different effector subsets. The main subsets currently distinguished are: TH1, TH2, TH17 and several regulatory T cell subsets with inhibitory activity that limit the extent of immune activation [16], [18].

### *2.2-1. CD8+ T cells*

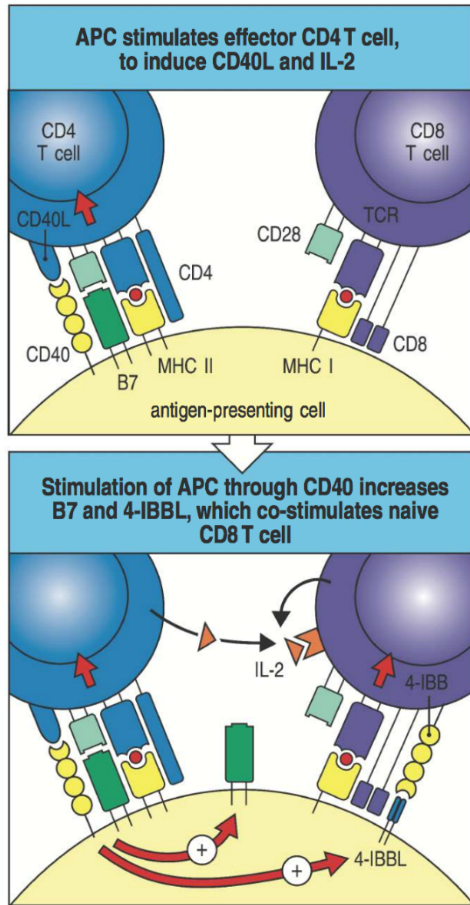
CD8+ T cells are very important for immune defense not only against intracellular pathogens, including viruses and bacteria, but also against tumors. The infected cells display fragments of pathogen-derived proteins as peptide-MHC class I complexes on their surface that are recognized by the activated CD8+ T cells or CTLs. When a CD8+ T cell recognizes its antigen and becomes activated, it has two major mechanisms to kill infected or malignant cells. The first major function is the production and release of cytotoxic granules that contain two families of proteins, perforin, and granzymes. Perforin forms pores in the membrane of the target cell that allow the granzymes to enter the infected cell, cleaving and activating a family of proteins (caspases) that trigger cell death by apoptosis (Scheme 2, A).

The second major way for CTLs to destroy infected cells is via Fas/FasL interactions. Activated CD8+ T cells express FasL on their cell surface, which binds to its receptor, Fas, on the surface of the target cell. This binding causes the Fas molecules on the surface of the target cell to trimerise, which pulls together signaling molecules resulting in the activation of the caspase cascade and consequent apoptosis of the target cell (Scheme 2, B) [16], [17], [18].




**Scheme 2** Mechanism of CTL mediated toxicity (from Cellular and Molecular Immunology, 8th edition)

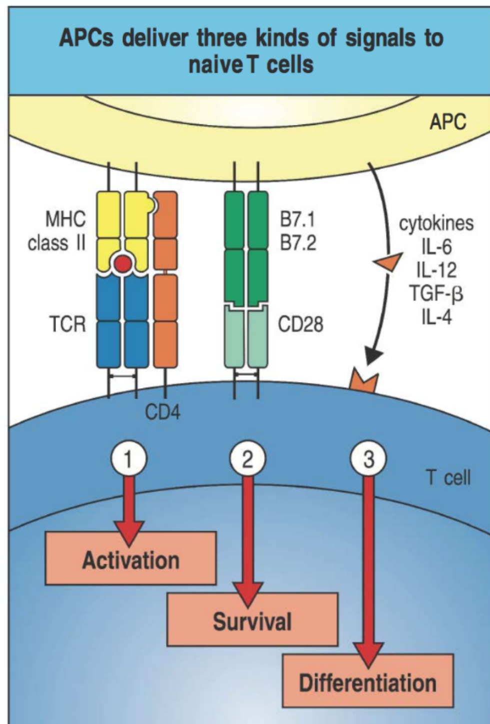
The priming and activation of naïve CD8+ T cells happens usually after the recognition of a pathogenic peptide in context of MHC-I on the surface of APCs (Scheme 3). In most instances, CD8+ T cell activation requires crucial help provided by CD4+ effector T cells. Help is provided mainly *via* the production of a specific class of hormones, designated cytokines needed to amplify the stimulation and activate the proliferation of both CD8+ and CD4+ T cells. In this context CD8, CD4 and dendritic cells further interact via co-stimulatory molecules within the same microenvironment. Upon interaction CD4+ T cells produce several cytokines among which the cytokine Interleukin-2 (IL-2) is the major driver of T cell proliferation and maturation (Scheme 3) [18].




**Scheme 3** Amplification of CD8+ T cell response by CD4+ T cell (from Janeway Immunobiology, 8th edition)

### *2.2-2. CD4+ T cells*

The priming of naïve CD4+ T cells takes place also in the peripheral lymph nodes were the APCs migrate after catching and processing the pathogen's antigen from the periphery. The priming can be triggered in only one way that include 3 steps or signals: Signal 1 comprises the antigen-specific signal derived from the interaction of a specific peptide-MHC-II complex with the TCR and its co-receptor, the CD4 molecule. Then the survival and expansion of the naïve cell are promoted after the delivery of signal 2 by the co-stimulatory molecules. The best known co-stimulatory molecule is CD28, receptor of the B7 molecules that are expressed on the APCs surface. The final signal 3 is triggered by the different molecules and cytokines secreted by the APCs or by T cells. Depending upon the nature of these soluble molecules, naïve CD4+ T cells mature in distinct subsets of effector T cells (Scheme 4). For example, the differentiation into TH1 is triggered by the secretion of IL-12 by the APC and IFN-γ by the T cell, whereas the differentiation into TH2 is triggered mainly by IL-4 [16].




**Scheme 4** Priming of naive CD4+ T cells by 3 signals provided by APCs (from Janeway Immunobiology, 8th edition)

The distinct polarization of CD4+ T cells, THl, TH2, TH17, and the regulatory T cells (Treg) is largely defined on the basis of the different combinations of cytokines they secrete.

Briefly, TH1 cells produce different types of cytokines, essentially IFN-γ and IL-2, that activate phagocytosis and foster the development of cytotoxic lymphocytes that are the main effectors of cell-mediated immune response against viruses and tumor cells. In fact, IFN-γ also upregulates the expression of MHC-I and MHC-II molecules on a variety of cells especially the ones that are infected by or have ingested pathogens, thereby stimulating antigen presentation to T cell (Scheme 5, 1st panel). TH2 cells are specialized for promoting immune responses to parasites and also promote allergic responses. They provide help in B cell activation and secrete the B cell growth factors IL-4, IL-5, IL-9, and IL-13. The principal membrane-bound effector molecule expressed by TH2 cells is CD40L, which binds to CD40 on B cells and induces B-cell proliferation and isotype switching (Scheme 5, second panel). TH17 cells produce members of the IL-17 family and IL-6, and promote acute inflammation by helping the recruitment of neutrophils to sites of infection (Scheme 5, 3rd panel). $T_{reg}$ cells, of which there are several types, produce inhibitory cytokines such as IL-10 and TGFβ and exert inhibitory actions through different mechanisms (Scheme 5, 4th panel) [16].

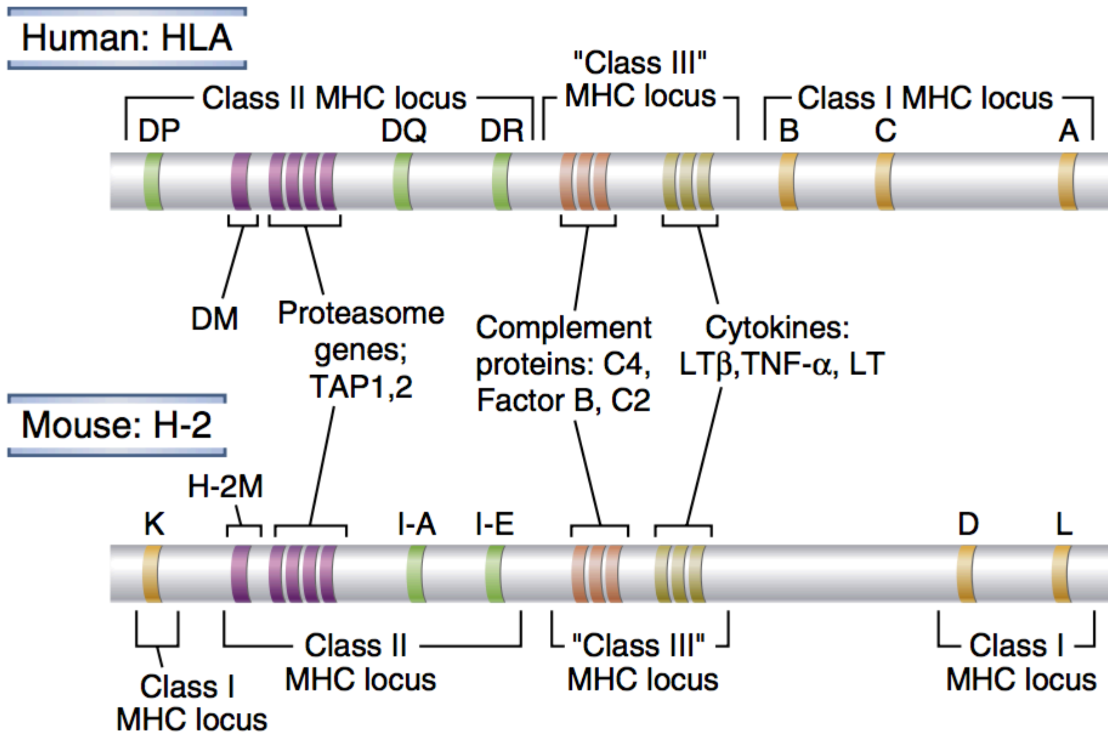
| CD4 T cells: peptide + MHC class II | | | | | | | |
|---|---|---|---|---|---|---|---|
| $T_H1$ cells | | $T_H2$ cells | | $T_H17$ cells | | $T_{reg}$ cells | |
| Macrophage-activating effector molecules | Others | Barrier immunity activating effector molecules | Others | Neutrophil recruitment | Others | Suppressive cytokines | Others |
| IFN-γ<br>GM-CSF<br>TNF-α<br>CD40 ligand<br>Fas ligand | IL-3<br>LT-α<br>CXCL2 (GROβ) | IL-4<br>IL-5<br>IL-13<br>CD40 ligand | IL-3<br>GM-CSF<br>IL-10<br>TGF-β<br>CCL11 (eotaxin)<br>CCL17 (TARC) | IL-17A<br>IL-17F<br>IL-6 | TNF<br>CXCL1 (GROα) | IL-10<br>TGF-β | GM-CSF |

**Scheme 5** The different subsets of CD4+ effector T cells and the molecules that they secrete or express (from Janeway Immunobiology, 8th edition)

# Part I.
# Chapter 2: Major Histocompatibility System and Antigen Presentation

As it was already mentioned, the T cells can only recognize antigens in the context of MHC class I or class II molecules on the surface of the cells. These proteins were first discovered as histocompatibility antigens considering their prominent role in the acceptance or rejection of a grafts from an unrelated donor. MHC class I and class II molecules are encoded by the MHC system that accommodates more than 100 distinct genes. It is designated as Human Leukocyte Antigen or HLA in human, and H-2 in mice. The MHC system has three major characteristics that contribute to its large diversity and complexity within the species: (1) it is extremely polymorphic; (2) is polygenic; (3) both haplotypes are co-dominantly expressed.

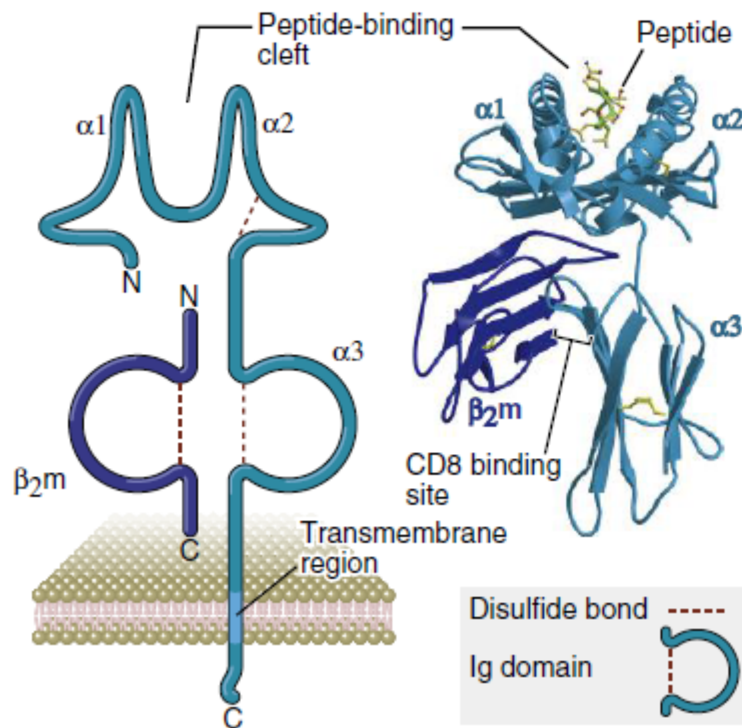
The genetic organization is similar in both human and mouse (Scheme 6). In human, there are three class I MHC genes called HLA-A, HLA-B, and HLA-C encoding the three types of MHC-I molecules and three subsets of MHC class II genes encoding the three distinct class II molecules HLA-DP, HLA-DQ, and HLA-DR. Similarly, in mice, three genes H-2K, H-2D, and H-2L encode class I MHC proteins K, D and L that are homologous to human HLA class I genes. On the other hand, mice have only two expressible MHC-II loci that encodes the I-A and I-E MHC-II proteins (Scheme 6) [16], [18], [19].




**Scheme 6** Gene structure of human and mouse MHC (from Cellular and Molecular Immunology, 8th edition)

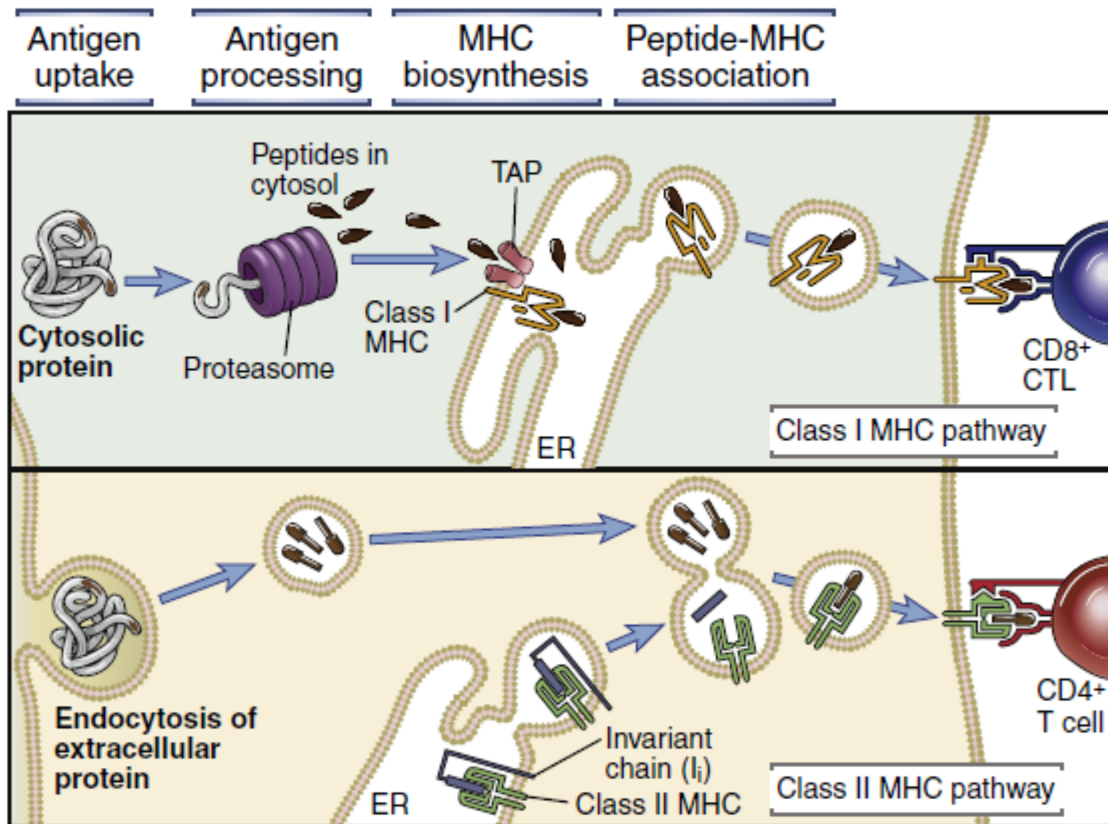
## *1. MHC-I molecules*

MHC-I molecules are composed of two non-covalently linked polypeptide chains, an MHC-encoded α heavy chain of 44 to 47 KDa and a non-MHC-encoded β2-microglobulin of 12 KDa (Scheme 7). The heavy chain consists of three extracellular domains α1, α2, and α3 which are about 90 amino acids long each. The antigen-binding groove or cleft of MHC-I is confined to the α1 and α2 domains including most of the polymorphic aminoacid residues. The α3 chain is conserved among all class I molecules and folds into an immunoglobulin (Ig)-like domain that contains the binding sites for CD8 molecule expressed on T cells and for the β2m. The β2m is crucial for MHC-I expression on the cell surface. Mice having their β2m gene mutated do not express MHC-I molecules and are severely defective in antigen presentation to T cells [18], [19].




**Scheme 7** The structure of MHC class I molecule (from Abbas, Cellular and Molecular Immunology,8$^{th}$ edition)

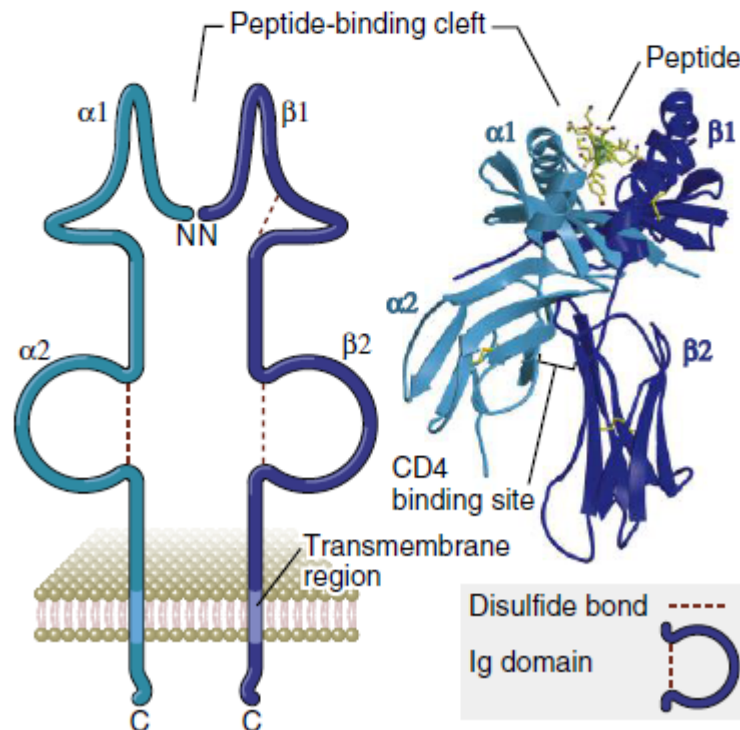
MHC-I molecules are expressed on all nucleated cells and are responsible for presenting peptides from processed proteins of cytosolic and nuclear origin at the surface of the cell (Scheme 8). These proteins are tagged for degradation in the proteasomes and should translocate into the endoplasmic reticulum (ER) through a molecular heterodimer designated TAP (transporter associated with antigen presentation) in order to be scrutinized for binding to MHC-I molecules. In the ER, MHC-I molecules are bound to chaperone-like proteins such as calreticulin or Tapasin that are essential for the stability of the molecule. Once they interact with the complementary peptide, usually 8-9 aminoacid long, MHC class I molecules are exported outside the ER, undergo post-synthetic modifications, such as glycosylation, in the Golgi and finally migrate to the cell surface [20], [21].




**Scheme 8** Pathways of antigen processing and presentation (from Abbas, Cellular and Molecular Immunology, 8th edition)

## 2. *MHC-II molecules*

MHC-II molecules are composed of two non-covalently linked polypeptide chains an α chain of 34 to 36 KDa and a β chain of 26 to 32 KDa, that, unlike MHC-I molecules, are both MHC encoded. The polymorphic residues of both chains reside in the α1 and β1 domains. These domains form the peptide binding cleft that is open at both ends and for this reason it may accommodate longer peptides, up to 30 amino acids, with respect to those bound to MHC class I molecules. The α2 and β2 segments of class II molecules, similar to class I α3 and β2-microglobulin, are folded into Ig domains, are non-polymorphic and contain the binding site for the CD4 molecule (Scheme 9) [18], [19].




**Scheme 9** Structure of a class II MHC molecule (from Cellular and Molecular Immunology, 8th edition)

Unlike MHC-I molecules, MHC-II have a restricted cell type expression. They are expressed mainly on dendritic cells, macrophages, and B cells. Under certain functional stimuli, as for example by IFN-γ, they may be expressed in other cell types, including epithelial and endothelial cells. They are in charge of presenting exogenous proteins to CD4+ T cells (Scheme 8). The initial step in the exogenous antigen presentation pathway of MHC-II is the internalization of the exogenous proteins in endosomal vesicles where they are degraded. Meanwhile, in the endoplasmic reticulum, MHC-II molecules are assembled from an α and a β chain and associate with the invariant chain to prevent the binding of proteins present in the endoplasmic reticulum to the peptide-binding cleft. The alpha-beta-invariant chain complexes are then routed to the endosome-lysosome compartment where they find the digested proteins derived from exogenous sources. Inside the fused vesicle, the same proteolytic enzymes that degraded the internalized protein digest the invariant chain leaving a 24-amino acid remnant referred to as the class II-associated invariant chain peptide (CLIP). The CLIP is then removed allowing the binding of the 30-amino acid long peptide in the MHC-II peptide-binding cleft. The MHC-II-peptide complex is finally transported to the cell surface where the exposed peptide can undergo CD4+ T cell scrutiny [18], [21].

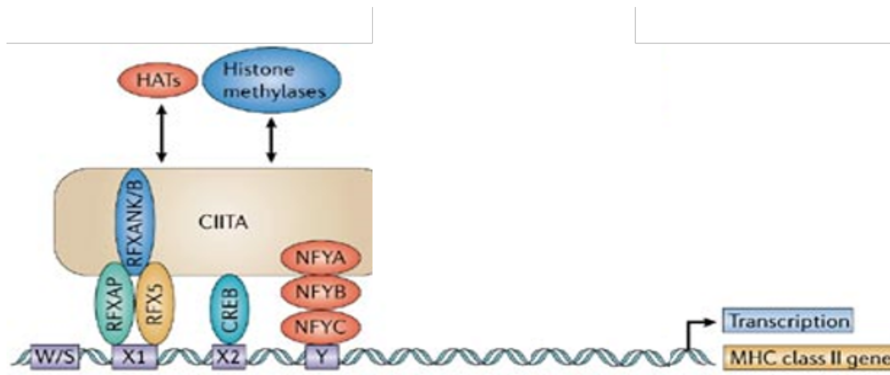
### *2.1- MHC-II molecules and CIITA*

The expression of MHC-II gene expression is tightly regulated at the transcriptional level. The major controller of this expression is a master regulator, designated class II transactivator (CIITA) [22], [23]. The identification and characterization of CIITA was accomplished after the generation of a somatic cell mutation that resulted in a MHC-II negative gene expression in RJ 2.2.5 human B cells [24]. The mutant phenotype was complemented by somatic cell hybridization with murine MHC-II positive cells revealing the presence of a dominant locus encoding the trans-acting activator and acting across species barriers. This locus was mapped to mouse chromosome 16 and designated *Air-1* [25], [26], [27]. Subsequently, the product of the *Air-1* human equivalent gene was cloned by a gene complementation approach and was referred to as CIITA [28].

CIITA transcription itself is controlled by a large regulatory region that contains four distinct promoters identified as pI, pII, pIII and pIV [29]. Promoters pI, pIII and pIV of CIITA gene are highly conserved in mouse and human; however, an equivalent promoter for pII has not been identified in mice and its function in human is not acknowledged yet. Each promoter controls the expression of CIITA in a cell specific manner. Early studies indicated that pI is responsible for CIITA transcription in DCs, pIII was specifically active in B cells, and pIV is IFN-γ induced [29], [30]. Several subsequent studies revealed that pI promoter is a myeloid cell specific promoter that is responsible of the expression of CIITA in conventional DCs and IFN-γ activated macrophages. The promoter pIII is active in lymphoid cells, B and T cells in addition to plasmacytoid DCs. The promoter pIV is important in CIITA expression in thymic epithelial cells [31].

Once CIITA is expressed, it controls the transcription of MHC II genes by binding to DNA-binding proteins, which themselves bind to DNA sequences 150 to 300 base pairs upstream the transcription initiation site (Scheme 10). This regulatory module designated SXY consists of four DNA sequences, S, X1, X2 and Y boxes. Moreover, the SXY is also found upstream accessory genes needed for MHC-II function, such as the invariant chain. The RFX (regulatory factor X consisting of RFX5, RFXANK-RFXB and RFXAP) protein complex binds to the X1 box of the SXY; CREB (cyclic AMP response element binding protein) interacts with the X2 box and NF-Y (nuclear factor Y consisting of NF-YA, NF-YB and NF-YC) binds to the Y box. All these DNA binding proteins interact with CIITA to regulate MHC-II transcription. CIITA is also

capable of recruiting histone methylases and histone acetyl-transferases (HATs) to enhance gene activation [31].

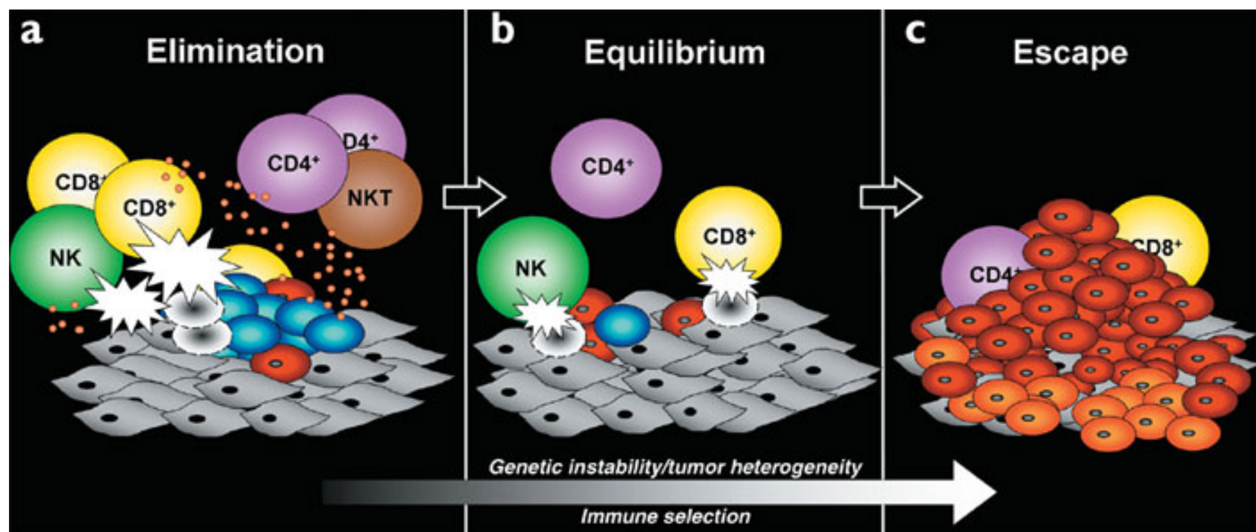



**Scheme 10** Mechanism of Action of CIITA (from Hoffman et al, 2006 Nature Reviews)

# Part I.
# Chapter 3: Cancer Immunosurveillance, Escape and Immunotherapy

Cancer has thrived to be one of the most hazardous health problems worldwide leading to high morbidity and mortality rates in adults and in children as well. The onset, expansion, persistence and spreading of tumors are under the control of a complex series of events that encompass both intrinsic modifications of cancer cells (such as genetic mutations in proto-oncogenes and in tumor suppressor genes, and alteration of the apoptotic process), which cumulatively impact on the homeostasis of the cell cycle, as well as extrinsic mechanisms related to the capacity of the host to counteract tumor growth. The concept that the immune system can protect the host by detecting and eliminating cancer cells has been widely debated over the years. Some discoveries show that indeed the immune system is important for destroying tumor cells; on the other hand, other evidences indicate that the immune system can also indulge tumor growth by favoring the escape of the most aggressive tumor cells that are capable of developing escape mechanisms from the immune response. These findings have led to the development of a refinement hypothesis of immunosurveillance termed cancer immunoediting hypothesis, which has been used to describe the host protective role and the tumor escape [18], [32], [33].

Immunoediting comprises three different phases: elimination, equilibrium and escape (Scheme 11). Elimination represents the host defense against tumor cells or immunosurveillance. Equilibrium embodies the period after the immune system failed to eliminate all tumor cells and escape refers to the growth of tumor cells that have successfully surpassed any immunological surveillance [32].




**Scheme 11** The three Es of cancer immunoediting (from Gavin P. Dunn et al. 2002, Nature Immunology)

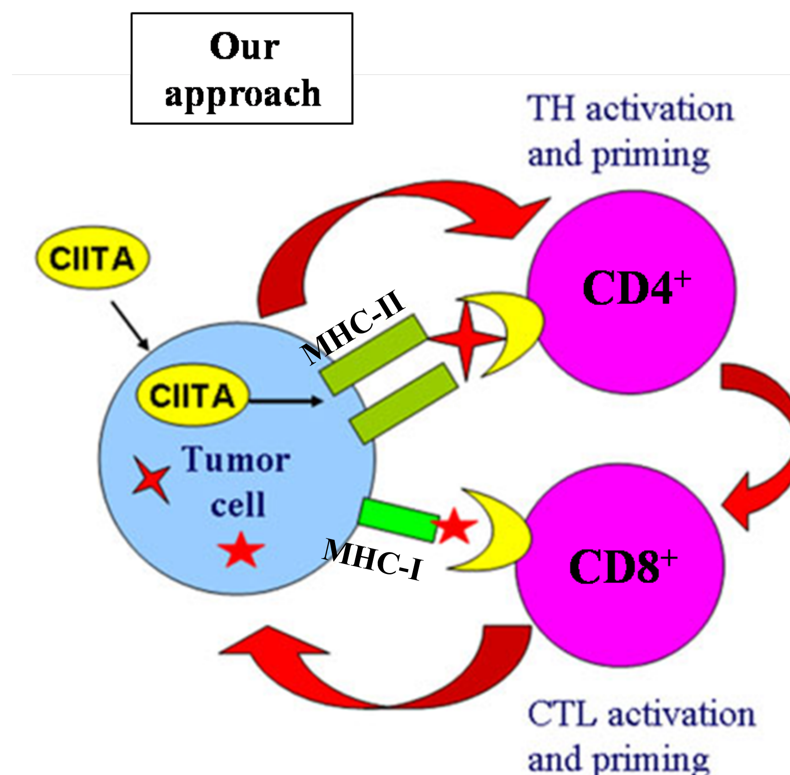
It is recognized that the mechanisms tumor cells use to escape the immune system lay in two basic categories: tumor intrinsic mechanisms associated with tumor cells and tumor-associated antigens, and tumor extrinsic mechanisms associated with the host immune system. The first group includes the lack of expression of MHC II molecules and costimulatory molecules, down regulation or loss of expression of MHC I molecules, downregulation of expression of genes associated with antigen processing and presentation such as TAP or β2m, low level of expression of tumor associated antigens at early phases of tumor growth, loss of antigenic peptides, physical barrier preventing effector cells accessing tumors, and loss of response to IFNs. The second group includes mechanisms of tolerance of T cells to tumor-specific antigens, suppression of T cells caused by tumor derived factors, defects in antigen presentation by professional APCs, and impaired APCs maturation [32].

It is therefore apparent that potential immunological approaches to counteract tumor growth and spreading have to be focused at eliminating and/or bypassing the above escape mechanisms. Many therapeutic strategies have been used in the past, such as peptide vaccination aimed at eliciting tumor specific CTLs, cytokine injection to rescue potential effector cells from latency and low replication, injection of DCs pulsed with class I restricted peptides.  However, none of these elicited the expected or hoped optimal recovery mostly because optimal triggering of CD4 TH cells was not targeted. As mentioned earlier in the introduction TH cells are crucial in all cellular and humoral immune responses. For instance, CD8+ T cell cannot be efficiently activated and no memory cell will be generated without a further help from CD4+ TH cell [4], [6], [7], [8].

# Part II.

## II.1- Our Approach and Previous Results

The approach of our laboratory is specifically directed to the optimal stimulation of tumor-specific CD4+ TH cells for their key role in leading a strong, long lasting adaptive immune response. In order to increase the triggering of tumor-specific TH cells we used a strategy that consists in a genetic modification of the tumor cells by stably transfecting them with the cDNA of CIITA. This modification induces the optimal and “physiological” expression of MHC-II molecules on tumor cell surface. The believe was that if MHC-II molecules can be expressed in tumor cells, these cells may function as surrogate APCs for their own TAA and directly prime and activate CD4+ TH cells and consequently enhance CTLs activation. The approach is summarized in the following scheme 12. Different tumor cell lines originated from distinct tissues and sharing the same H-$2^d$ genetic background were successfully used in this approach. Indeed, following this concept, it was demonstrated that CIITA-induced MHC class II-expressing tumor cells can be recognized *in vivo* and elicit both tumor-specific CTLs and, more importantly, tumor specific CD4+ TH cells [10], [11], [12].




**Scheme 12** Tumor Cell as Antigen Presenting Cells (from ACCOLLA and TOSI 2013, Journal of Neuroimmune Pharmacology)

## II.2- Aim of The Project

1. The prominent goal of my thesis project was to verify whether mice with a different genetic background such as the one represented by C57BL/6 (H-$2^b$ haplotype) can mount an adoptive immune response against CIITA-driven MHC-II positive tumor cells, similar to the one observed in Balb/c (H-$2^d$ haplotype) mice. If this takes place then our approach could be generalized not only to tumor cells of distinct histotypes, as previously shown [12], but also to distinct MHC haplotypes/genotypes, and give further support to extend our approach to human clinical setting.

2. The choice of the C57BL/6 mouse model was also further motivated by the fact that these mice have a defective I-Eα gene and therefore they can express only MHC class II I-A heterodimers and not I-E heterodimers [13]. Their repertoire of MHC class II-restricted antigens is thus qualitatively and quantitatively distinct from the one expressed by the Balb/c mice and this may represent an additional immunological diversity between the two mice strains to investigate the adaptive response against the tumor.

3. Moreover, and importantly, in the C57BL/6 mouse model, we could take advantage of the CD11c.DTR transgenic mouse system (H-$2^b$ haplotype), a mouse strain in which a conditional deletion of DCs can be induced. We could then verify whether CIITA-driven MHC-II positive tumor cells can be still recognized and rejected in absence of DCs. If this were the case, our hypothesis that CIITA-transfected MHC-II positive tumor cells act *in vivo* as APCs for TH cells priming could be unambiguously demonstrated.

# Part III.
# Chapter 1: Materials and Methods

## *1. Transfection and phenotypic analysis of tumor models*

MC38 colon carcinoma, a cell line of C57BL/6 H-$2^b$ haplotype from a female mouse, was cultured in Dulbecco's Modified Eagle Medium, DMEM, with 4.5 g/l Glucose, L-Glutamine and supplemented with 10% of heat-inactivated fetal calf serum (FCS) (Lonza BioWhittaker™, Catalog number: BE12-604F).

The full-length human CIITA cDNA was already inserted into the Xho-I site of the pLXIN retroviral vector (Clontech, Catalog number 63150). DNA transfections were performed using FuGENE™ HD Transfection Reagent (Promega™, Catalog number: E2312), following the manufacturer's instructions with a 1:3 ratio of µg DNA: µg of Transfection reagent respectively. MHC-II positive transfectants were sorted by magnetic beads and stable clones were selected by limited dilution cloning. The tumor cell line was also transfected with pLXIN empty vector as a control.

The expression of different immune markers was assessed on the surface of the transfected and parental cell lines by immunofluorescence and flow cytometry (BD FACSAria™ II Cell Sorter, BD Biosciences, San Jose, CA 95131 USA) using the following antibodies: *M1/42* anti-H2 class I (Biolegend®, Catalog number: 125508), *M5/114.15.2* anti-IA/IE (Biolegend®, Catalog number:107626), *16-10A1* anti-CD80 (Biolegend®, Catalog number:104705), *GL1* anti-CD86 (Becton Dickinson, Catalog number:558703) and *M1/70* anti CD11b (Affymetrix eBioscience, Catalog number:17-0112-82).

## *2. In vitro proliferation rate assay*

MC38-CIITA and MC38 cells were plated in triplicates in 24 wells plate, $5x10^4$ cells per well. Proliferation rate and cells viability were assessed using trypan blue and Neubauer Chamber every 24 hours for 3 days.

## *3. Vaccination and challenge*

7-9 weeks old C57BL/6 female mice (Charles River Laboratories Italia SRL, Calco, Italy) were subcutaneously (s.c.) injected with $5x10^4$ of MC38 parental or CIITA-transfected tumor cells, resuspended in 100 µl of RPMI (Lonza BioWhittakerTM, Catalog number: BE12-702F) without FCS. The expression of CIITA-driven MHC-II was confirmed the day of the injection by

immunofluorescence and flow cytometry as described above. Tumor growth along with the overall health condition of the mice were checked at least twice a week. The tumors were measured weekly using a caliper and registered in $mm^2$.

The mice that did not show any tumor growth after 5 weeks of their injection with CIITA-transfected tumor cells were challenged with a s.c. injection of $2x10^5$ parental tumor cells (four times the number of the first CIITA-expressing tumor cell injection).

Each experiment was repeated at least twice using 5-8 mice per group. All animal work has been conducted according to relevant national and international guidelines and was approved by the University of Insubria Internal Ethical Committee CESA (project 07-2013) and by the Italian Ministry of Health.

## 4. *Ex vivo MC38-CIITA tumors analysis*

MC38-CIITA outgrowing tumors were excised from mice and a single cell suspension was obtained from each tumor by dissociation with a sterile syringe plunger in a 70 µm cell strainer. The level of expression of MHC-I and MHC-II on the surface of the *ex vivo* tumor cells was assessed by immunofluorescence staining and flow cytometry analysis as described above.

## 5. *ELISA analysis*

Spleens were harvested from naïve mice, MC38 tumor-bearing mice and MC38-CIITA vaccinated mice originating from the same experiment. Single cell suspension was obtained from each spleen by dissociation with a sterile syringe plunger in a 70 µm cell strainer, then, after centrifugation for 15 minutes at 1500 rpm and discarding the supernatant, the cells were treated with Ammonium Chloride Potassium or ACK lysing buffer for 3 minutes on ice in order to lyse the erythrocytes. The obtained splenocytes from the different spleens were plated in a 48 wells plate in triplicate, at a concentration of $4x10^6$ cells /well. The supernatant was assessed for of the secretion of IFN-γ after 4 days of culture using Mouse IFN-γ Ready-SET-Go ELISA kit (Affymetrix eBiosceience, Catalog number: 88-7314) following the manufacturer' protocol.

## 6. *Adoptive cell transfer*

Spleens from challenged mice that did not show parental tumor growth after 4 weeks from injection were harvested and processed as mentioned above. A single cell suspension was obtained and used to purify CD4+, CD8+ T cells (Mouse T Cell Isolation Kit, Miltenyi Biotec GmbH, cat. number 130-095-248/130-090-859 respectively), or CD19+ B cells using Easy Sep

Mouse B Cell Enrichment Kit (STEMCELL Technologies$^{TM}$, catalog number: 19754). The purity of the purified cells was confirmed by flow cytometry using *145-2C11* anti-CD3e (Becton Dickinson, Catalog number: 553066), *RM4-5* anti-CD4 (Becton Dickinson, Catalog number: 550954), *53-6.7* anti-CD8a (Biolegend®, Catalog number: 100711) and *6D5* anti-CD19 (Biolegend®, Catalog number: 115511) antibodies.

Normal splenocytes were obtained from naïve C57BL/6 female mice of the same age. Naïve female mice, 7-9 weeks old, were s.c. co-injected with $2x10^5$ parental MC38 tumor cells, in a 100 µl volume of RPMI without FCS, along with either immune total splenocytes, CD4+ T cells, CD8+ T cells, CD19+ B cells, or total naïve splenocytes as a control, in a tumor cells:immune cells ratio of 1:50, 1:15, 1:10, 1:25 and 1:50 respectively. The experiment was repeated at least twice using 5-8 mice per group. The mice under experimentation were followed for 4 to 5 weeks and the data were recorded as described before.

### 7. *Dendritic cells depletion in CD11c.DTR transgenic C57BL/6 mice*

CD11c.DTR heterozygous C57BL/6 mice (kindly provided by Dr. N. Garbi, University of Bonn, Germany) express Diphtheria Toxin Receptor (DTR) under the control of the CD11c gene promoter that is highly expressed in DCs. Depletion of DCs (CD11c+/MHC-II+) can be obtained by injecting these mice with Diphtheria Toxin (DT), whose cytotoxicity is strictly dependent on receptor-mediated endocytosis [15]. CD11c.DTR heterozygous transgenic male mice were bred with wild type (WT) C57BL/6 females purchased from Charles River Laboratories. Genotyping was performed on the offsprings in order to select the transgenic progeny. The DNA was extracted from tail-cuts and PCR was done for the β2 microglobulin (βm) as a reference gene and for the Ovalbumin (OVA) as a reporter gene located downstream the DTR gene under the control of the same CD11c gene promoter. The following primers were used in PCR:

OVA1 5'-AACCTGTGCAGATGATGTACCA-3'
OVA2 5'-GCGATGTGCTTGATACAGAAGA-3'

βm 1 5'-CACCGGAGAATGGGAAGCCGAA-3'
βm 2 5'-TCCACACAGATGGAGCGTCCAG-3'

Primers were obtained from Tib Molbiol Srl, Genova, Italy. Following the protocol established by Hochweller et al., [15] the selected transgenic female mice (7-9 weeks old) were daily injected intraperitoneally (i.p.) with DT (SIGMA-ALDRICH, Catalog number: D0564) for 11 days

starting 48 hours prior to tumor injection. Each mouse was injected with 8 ng/gbw of DT in a 100 µl volume of RPMI without FCS. DCs depletion was confirmed by immunofluorescence and flow cytometry analysis of CD11c+ cells, using the *HL3* anti-CD11c antibody (Becton Dickinson, Catalog number: 561044), and the *M5/114.15.2* anti-IA antibody on splenocytes isolated from transgenic mice sacrificed 72 hours after their first DT injection and compared to splenocytes of wild type mice.

$5x10^4$ MC38-CIITA tumor cells were s.c. injected in DT treated and untreated CD11c.DTR C57BL/6 female mice. Each group consisted of at least 5 mice. The experiment was repeated three times. The mice under experimentation were followed for 4 weeks and the data were recorded as described before.

## 8. Macrophages depletion with liposomal Clodronate

250 µl of 5 mg/ml Liposomal Clodronate (LClo) (http://www.clodronateliposomes.org), a potent anti-macrophage agent that upon phagocytosis induces an irreversible damage of the cell which then dies by apoptosis [34], were injected i.p. every 3 days for 2 weeks starting 4 days before tumors injections. Macrophages depletion was confirmed by flow cytometry analysis of peritoneal and spleen cells stained with *BM8* anti-F4/80 antibody (Biolegend®, Catalog number:123107) and the *M5/114.15.2* anti-IA antibody, on the following day of the second LClo injection.

Equal number ($5x10^4$) of MC38-CIITA tumor cells were s.c. injected in LClo-treated and untreated C57BL/6 female mice. Each group contains at least 5 mice. The experiment was performed two times. The mice under experimentation were followed for 4 weeks and the data were recorded as described before.

## 9. Statistical analysis

The statistical analyses were carried out using GraphPad Prism 6 and Student t-test was run to determine the significance. The results were considered significant if p values were $< 0.01$.

# Part III.
# Chapter 2: Results

## *1. CIITA-transfected MHC-II positive MC38 clone generation*

As shown in previously published work using cell lines from different histotypes of the H-$2^d$ haplotype [12], the transfection of CIITA triggered the expression of MHC-II (or I-A) also in the MC38 colon carcinoma cell line of H-$2^b$ haplotype. The cells were stably transfected and different steps of sorting by magnetic beads and limited dilution cloning were made in order to obtain a single stable CIITA-transfected MHC-II positive clone. The MHC-II expression during these steps was assessed by immunofluorescence and the selected clone was further used in the experiments (Fig. 1).

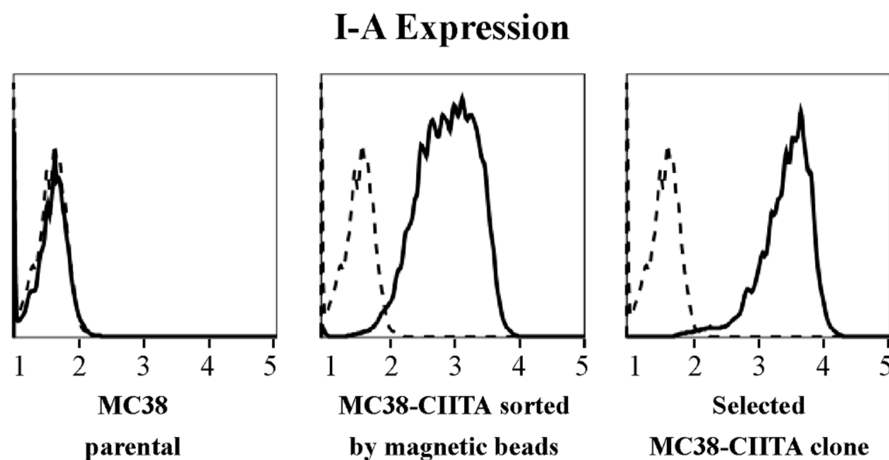



Fig. 1 **MHC-II positive MC38-CIITA clone generation**. The expression of MHC-II (I-A) on the surface of MC38 tumor cells was assessed at different steps after their stable transfection with CIITA. Immunofluorescence followed by flow cytometry analyses were done using the specific antibodies. From the left to the right panel we can see, first the MC38 parental cells that were negative for MHC-II; second the CIITA-transfected bulk of cells after being sorted by magnetic beads and being all positive for MHC-II; third the selected single clone after limited dilution cloning of the previous bulk and presenting a stable high level of expression of MHC-II. Results are expressed as number of cells (ordinate) versus the mean of fluorescence intensity (m.f.i.) in arbitrary units (abscissa). In each histogram, negative controls, obtained by staining the cells with an isotype-matched antibody, are depicted as dashed lines.

Moreover, MC38-CIITA stable clones underwent different studies in order to study the effect of the expression of CIITA on the cells.

Firstly, the stable clones were assessed for their expression of costimulatory molecules such as CD80 and CD86 that are involved in the MHC-II restricted antigen presentation. Results indicated that MC38-CIITA tumor cells were negative for these two markers, just like the parental tumor cells (Fig. 2).

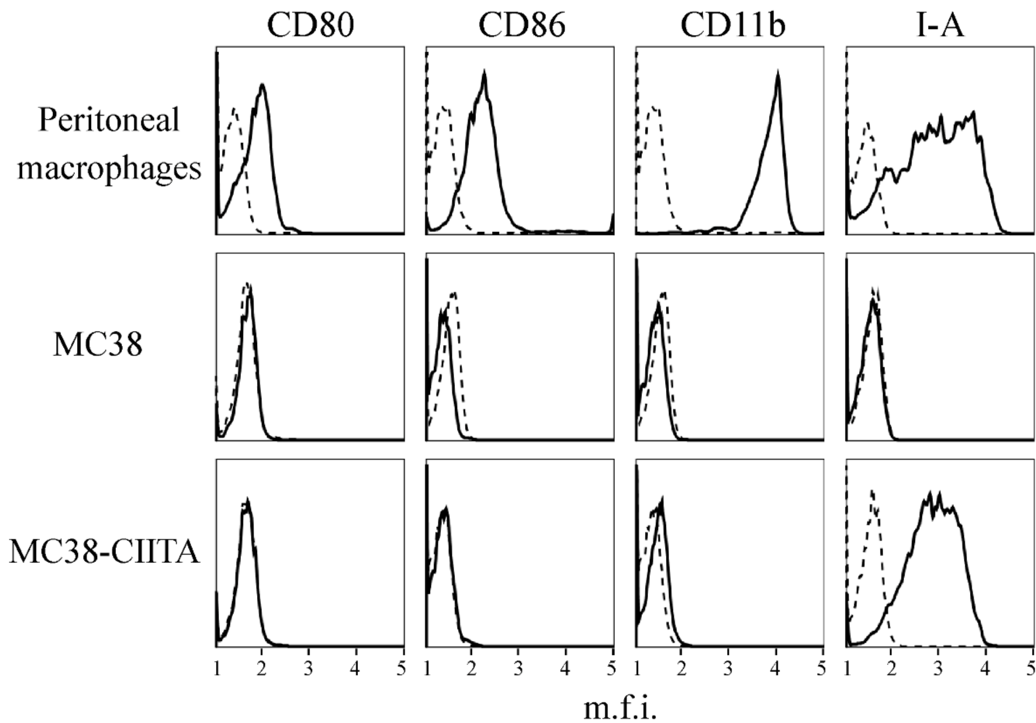



Fig. 2 **Lack of the expression of CD80 and CD86 costimulatory molecules on both parental and CIITA transfected tumor cells.** The expression of CD80 and CD86 co-stimulatory molecules (first two rows of vertical panels) was assessed on parental MC38 and MC38-CIITA tumor cells by immunofluorescence and flow cytometry and with the corresponding specific antibodies. The cells were also assessed for the expression of CD11b and MHC-II (I-A) markers (third and fourth row of vertical panels, respectively). As positive control for the expression of the 4 markers, a population of activated peritoneal macrophages from C57BL/6 mice was analyzed. Results are expressed as number of cells (ordinate) versus the mean of fluorescence intensity (m.f.i.) in arbitrary units (abscissa). In each histogram, negative controls, obtained by staining the cells with an isotype-matched antibody, are depicted as dashed lines.

Secondly, a proliferation test was performed on the stable transfected MC38-CIITA clone in comparison with the parental MC38 in order to evaluate if the CIITA expression affected the proliferation rate of the cells *in vitro*. No significant difference was detected between the proliferation rate of the CIITA-transfected and parental cells during three days of culture *in vitro* (Fig. 3).

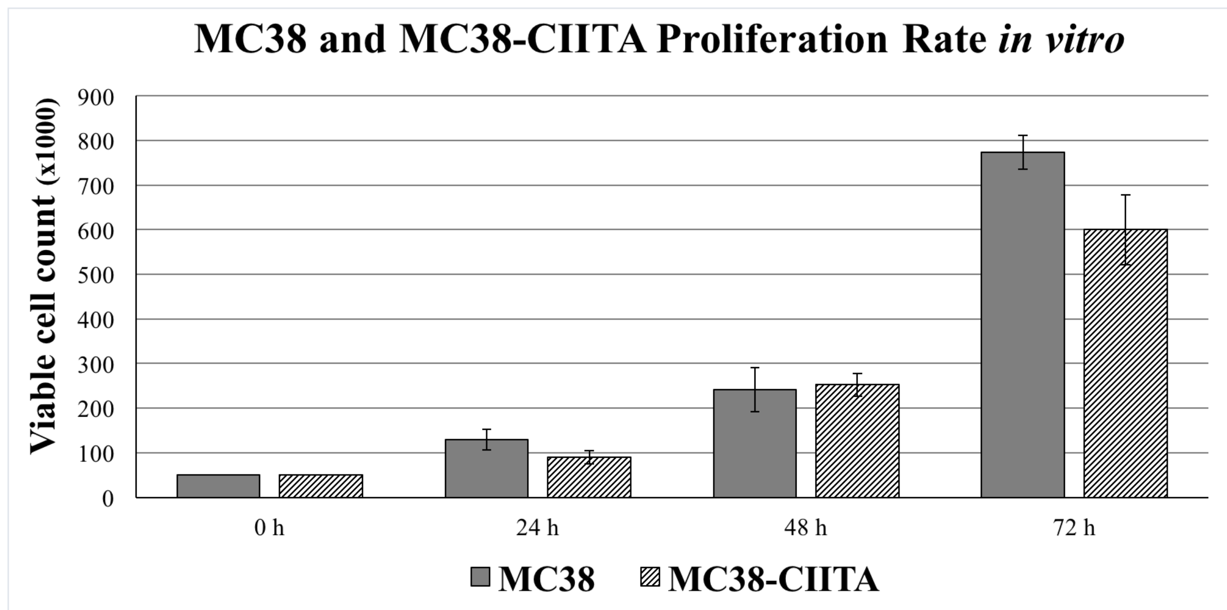



Fig. 3 **No effect for CIITA gene expression on the proliferation rate of MC38-CIITA.** The average number of viable cells was calculated from the three plated wells for each cell type at different time points and presented (ordinate) over time in hours (abscissa).

## 2. *CIITA-driven MHC-II+ tumor cells are rejected or strongly retarded in their growth*

Following the preliminary *in vitro* studies we moved to the *in vivo* ones in order to investigate the effect of CIITA expression on the anti-tumor immune response of the mice. MC38-CIITA and MC38 parental tumor cells were injected into naïve syngeneic C57BL/6 mice and tumor growth was monitored over time. MC38-CIITA tumor cells were either completely rejected or showed a significant retardation in their growth *in vivo* in comparison with their parental untransfected counterpart. In fact, after 5 weeks, 50% of the mice injected with MC38-CIITA did not develop any tumor (Fig. 4; a), and those that developed tumors displayed a growth kinetics 4 to 5 fold reduced as compared to the parental tumor growth that occurred in 80% of the injected mice (Fig. 4; b).

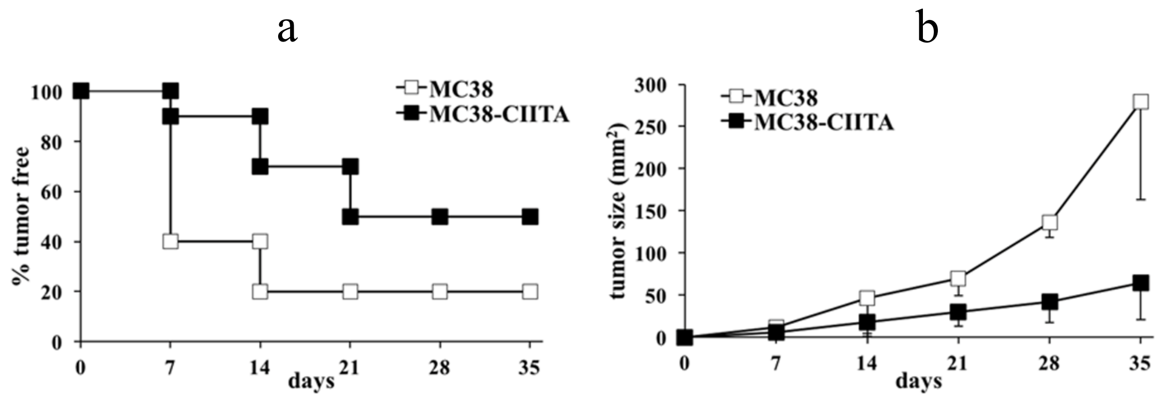



Fig. 4 **MC38-CIITA tumor cells are rejected or drastically retarded in their growth *in vivo*.** Equal number ($5x10^4$) of either parental MC38 or MC38-CIITA tumor cells were injected subcutaneously (s.c.) in 7-9 weeks old C57BL/6 female mice (at least 5 mice per group). Tumors growth along with the overall health condition of the mice were checked at least twice per week. (a) The percentage of tumor-free mice in both groups (ordinate) is presented as a function of time in days (abscissa). (b) The average size of tumors growing in each group is presented in $mm^2$ (ordinate) as a function of time in days (abscissa). Data represent means from more than two different experiments. Open symbols refer to parental tumor cells; full symbols refer to CIITA-transfected tumor cells. Differences between both groups in the graph of average tumor size kinetics were significant ($p$ value $< 0.01$) at all-time points.

### *3. MC38-CIITA tumor cells trigger a long lasting adaptive immune response*

To investigate whether the rejection and/or the growth retardation of MC38-CIITA tumors was due to an adaptive immune response and in order to further understand this response, we first challenged with parental MC38 cells the protected mice that did not show any tumor growth for five weeks after being injected with MC38-CIITA. The challenge was done by injecting four times the number of cells that was used to vaccinate the mice with MC38-CIITA. Interestingly, 100% of the challenged mice were protected and did not show any tumor growth compared to naïve mice that all display tumor growth after 2 weeks (Fig. 5; a, b). These results clearly demonstrate the acquisition of a long-lasting specific immune response not only against the immunizing CIITA-positive, MHC-II-positive tumor but also against the MHC-II-negative parental tumor.

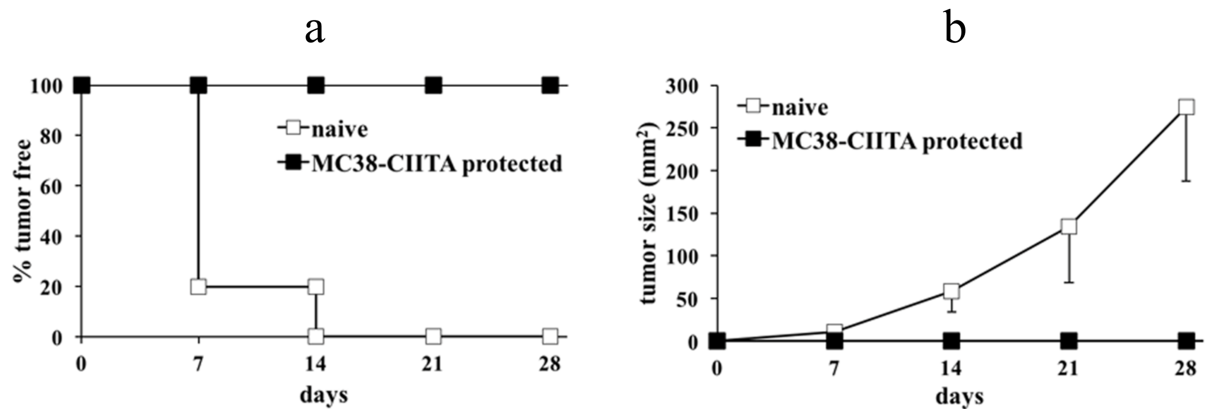



Fig. 5 **Vaccination with MC38-CIITA induces long lasting immunity against MC38 parental tumor.** Mice resistant to MC38-CIITA (MC38-CIITA protected) tumor growth or naïve control mice were challenged with $2x10^5$ parental tumor cells. (a) The percentage of tumor-free mice is presented (ordinate) as a function of time in days after injection (abscissa). (b) The average size in $mm^2$ of the tumors growing in each group is presented (ordinate) as a function of time in days after injection (abscissa). Differences between both groups in the graphs of average tumor size kinetics were highly significant (p value < 0.001) at all-time points

## *4. Loss of MHC-I and MHC-II by the MC38-CIITA growing tumors*

In order to understand why some mice developed tumors after injection of CIITA-transfected MC38 cells, freshly excised tumors from the MC38-CIITA tumor-bearing mice were assessed for their expression of MHC-I and MHC-II cell surface molecules by immunofluorescence and FACS analysis. Interestingly, these tumors presented with a loss of MHC-I expression and more importantly with a strongly reduced MHC-II expression as well, indicating that they underwent immunoediting *in vivo* to escape recognition by the immune system (Fig. 6).

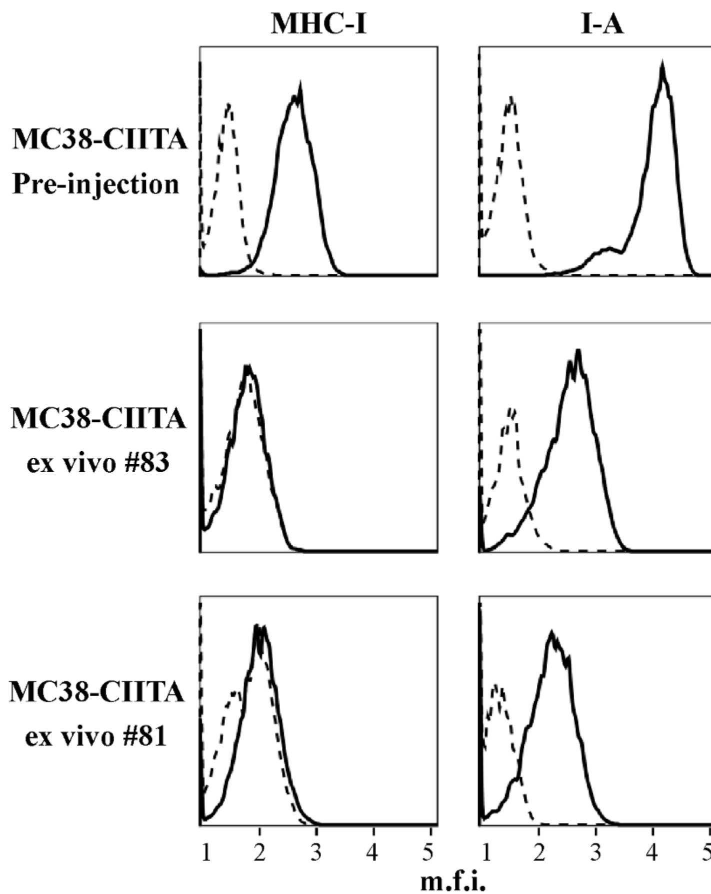



Fig. 6 **Loss of MHC-I and low level of MHC-II expression on the *ex vivo* MC38-CIITA tumors.** The expression of MHC-I and MHC-II was assessed by flow cytometry on the surface of MC38-CIITA *ex vivo* tumor cells at the day of the excision from the tumor bearing mice. The level of expression of MHC-I and MHC-II on the tumor cells at the day of the injection (First horizontal row) and on the excised tumors number 83 and 81 respectively after 4 weeks of the injection (Second and third horizontal row). Results are expressed as number of cells (ordinate) versus the mean of fluorescence intensity (m.f.i.) in arbitrary units (abscissa). In each histogram, negative controls, obtained by staining the cells with an isotype-matched antibody, are depicted as dashed lines.

### 5. *High IFN-γ secretion by splenocytes of immune mice*

Beside the excised tumors, spleens were also harvested and IFN-γ secretion from splenocytes excised from naïve mice, MC38 tumor bearing mice or MC38-CIITA protected mice was assessed by ELISA. Results showed a high secretion of IFN-γ by the splenocytes of immune mice, about 1500 pg/ml, in comparison with a much lower level of IFN-γ secretion by naïve or tumor bearing mice splenocytes, around 500 pg/ml (Fig. 7). The high level of IFN-γ secretion reflect a strong activation of the immune system of the MC38-CIITA protected mice and favors a cellular immune response mediated by CD4+ TH1 subset.

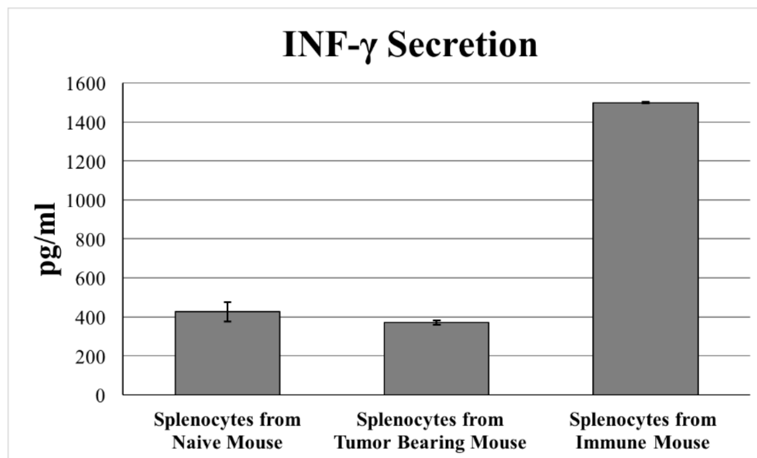



Fig. 7 **Immune activated splenocytes from MC38-CIITA vaccinated mice secrete high level of IFN-γ.** ELISA analyses were performed on the supernatant of $4x10^6$ splenocytes from naïve, immune or tumor bearing mice put in culture for 4 days. Each culture was made in triplicates and the ELISA wells were made in duplicates.

## *6. The long lasting immune response triggered by MC38-CIITA is orchestrated by CD4+ T cells*

The previously presented results clearly demonstrated that the vaccination with CIITA-driven MHC-II positive MC38 tumor cells triggers a long lasting adaptive anti-tumor immune response. To identify the immune cells responsible of the tumor rejection in vaccinated animals, total splenocytes, purified CD4+ T cells, CD8+ T cells or B cells of vaccinated and challenged mice were used in adoptive cells transfer (ACT) experiments.

As shown in Fig. 8, total splenocytes from MC38-CIITA protected and challenged mice (immune mice) confer protection to 100% of the injected mice against the tumor growth. Purified CD4+ T cells from immune mice were also efficacious in preventing tumor growth of MC38 parental tumor since 80% of the animals were protected (Fig. 8; a) and the remaining 20% presented a retarded growth (Fig. 8; b). Conversely, neither protection nor retardation of tumor growth was observed by injection of spleen cells from naïve animals or of purified B cells from vaccinated animals. Surprisingly, purified CD8+ T cells were only partially effective in preventing tumor growth, as only 20% of the animals were protected and the remaining 80% did not displayed potent growth retardation, suggesting that the primed CD8+ T cells are not sufficient to trigger a potent immune response against the parental tumors (Fig. 8; a and b). These findings are in contrast to that observed in the Balb/c system, in which both CD4+ and CD8+ T cells act equally well as effector immune cells against the tumors [12].

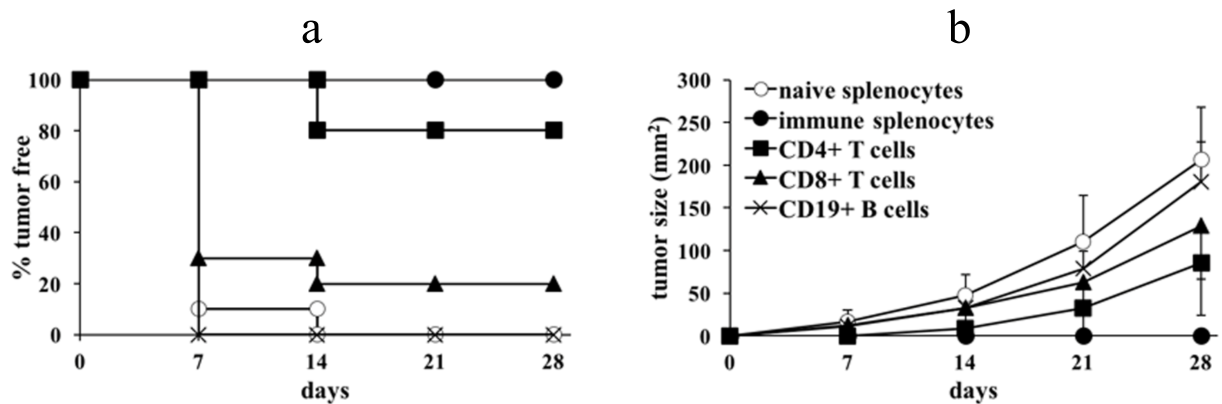



Fig. 8 **CD4+ TH cells are the key cells responsible for the anti-tumor immunity induced after MC38-CIITA vaccination.** Spleens from protected mice were isolated and processed to purify CD4+, CD8+ T cells, and B cells. Naïve mice were co-injected s.c. with $2x10^5$ MC38 parental cells together with either immune total splenocytes, purified CD4+ T cells, CD8+ T cells, CD19+ B cells, or with total naïve splenocytes, as a control. (a) The percentage of tumor-free mice in each group is presented in (ordinate) as a function of time in days after co-injection (abscissa). (b) The average size in $mm^2$ of tumors growing is presented (ordinate) as a function of time in days after co-injection (abscissa). No statistical significance was observed in the growth of tumors after adoptive cell transfer of naïve splenocytes and immune CD19+ B cells in MC38 group. Statistical significance was observed between naïve splenocytes or CD19+ B cells and immune splenocytes (p value < 0.01) and CD4+ T cells (p value < 0.01) at all time points, and with CD8+ T cells (p value < 0.01) after 21 days.

## 7. *CIITA driven MHC-II positive MC38 tumor cells act as surrogate APCs for priming and activating CD4+ TH cells*

The results presented in Fig. 8 clearly show that CD4+ TH cells were the responsible lymphocyte subpopulation capable of transferring anti-tumor protection to naïve mice. In order to assess the direct role of the CIITA-driven MHC-II expressing tumor cells as surrogate antigen presenting cells *in vivo*, we took advantage of a recently described transgenic mouse model, the CD11c.DTR C57BL/6 mice expressing the DTR gene along with the OVA gene downstream the promoter of CD11c gene that is highly expressed in DCs. In this system, DCs, believed to be the crucial cells for T cell priming, can be transiently depleted *in vivo* by injecting the mice with DT, offering the possibility to study an anti-tumor immune response in absence of this crucial APCs population [15].

CD11c.DTR mice were first selected after extracting DNA from their tail cuts and performing PCR in order to detect the presence of the OVA (Fig. 9).

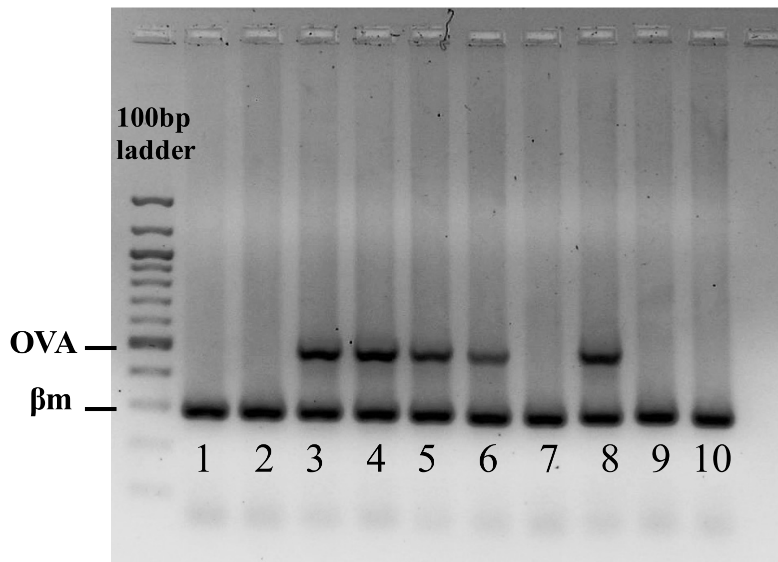



Fig. 9 **CD11c.DTR mice genotyping.** PCR samples were loaded on 2% agarose gel with Ethidium Bromide staining, and DNA was visualized under ultra violet exposure. Mice number 3, 4, 5, 6 and 8 are transgenic since they express the reporter OVA gene. βm is the reference or housekeeping gene that is present in all the samples.

The detected CD11c.DTR mice, were injected with DT following the protocol described in Materials and Methods. The kinetics of the MC38 and MC38-CIITA tumor take and tumor growth was followed over time. DCs depletion in tumor-injected mice was assessed by immunofluorescence followed by flow cytometry of spleen cells co-stained with anti-CD11c and anti-MHC (I-A) antibodies, since the most prominent DCs population displaying APCs activity expresses both these markers. Indeed, DT treatment of CD11c.DTR transgenic mice was able to virtually deplete the entire DCs population from the spleen (Fig. 10; a).
After DCs depletion 35% of mice injected with MC38-CIITA tumor cells fully rejected the tumor after 4 weeks from tumor cell injection. This proportion was similar to the one obtained in mice not undergoing DCs depletion (40%, Fig. 10; b, left panel). Moreover, the MC38-CIITA tumors that developed in DCs-depleted mice displayed a significant retardation in their growth and a strong reduction in their volume. This growth kinetics was indeed superimposable to that obtained in DT-untreated mice without DCs depletion (Fig. 10; b, right panel).
These results show that CIITA-tumor cells can still induce a potent immune response *in vivo* despite the absence of DCs the most prominent antigen presenting cells and the ones considered to be crucial for the initial priming of naïve antigen-specific CD4+ TH cells.

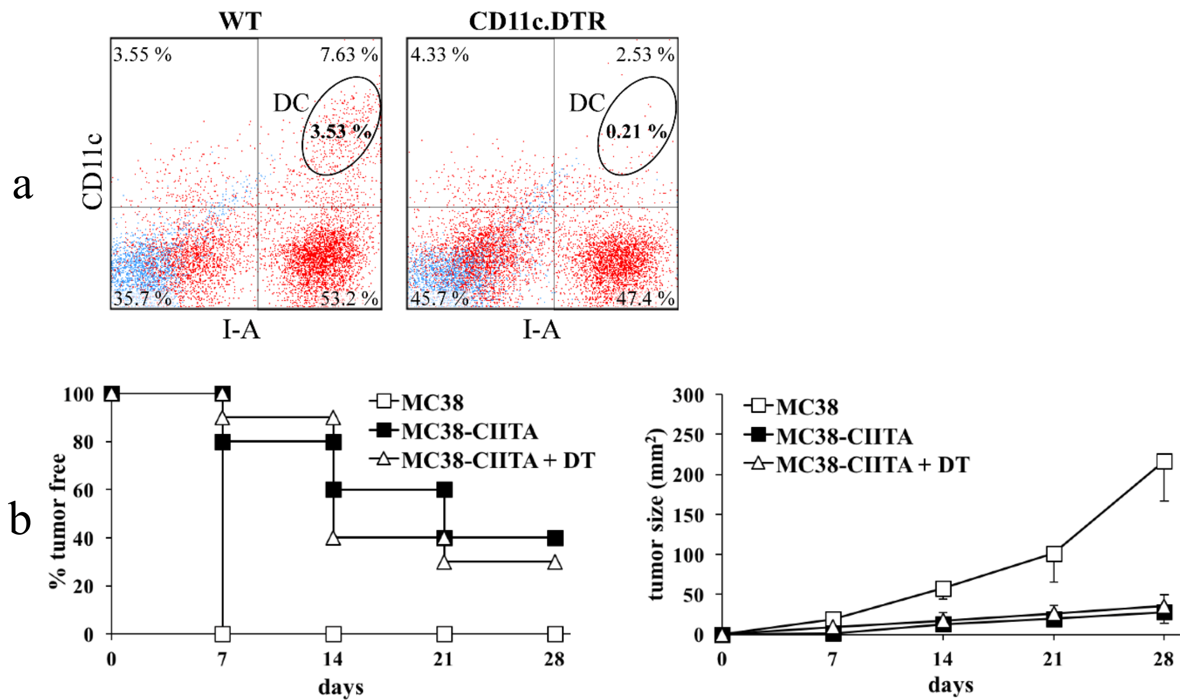



Fig. 10 **MC38-CIITA tumors cells are the only APCs (DCs Depletion).** (a) CD11c.DTR transgenic mice were treated with diphtheria toxin (DT) as described before. DCs depletion was checked after 3 days from the first DT injection by immunofluorescence and FACS analysis on spleen cells co-stained with anti-CD11c and anti- I-A directly labelled monoclonal antibodies and compared with the one in untreated mice. (b) Equal number ($5x10^4$) of MC38-CIITA tumor cells were injected in either untreated (MC38-CIITA) or DT-treated (MC38-CIITA + DT) CD11c.DTR transgenic mice. A group of untreated mice were also injected with the same number of parental MC38 cells, as control (MC38). Tumor take, measured as percentage of mice with tumors (ordinate, left panel) and kinetics of tumor growth (ordinate, right panel) were followed over time (days in abscissa).

In order to exclude the possibility that in absence of DCs other professional APCs, such as macrophages, could provide specific tumor antigen priming for naïve CD4+ T cells, CD11c.DTR transgenic mice were treated with liposomal clodronate (LClo), a chemical compound that is a potent anti-macrophage agent both *in vivo* and *in vitro*. In fact, after being phagocytosed by macrophages the clodronate is accumulated intracellularly and after exceeding a threshold concentration, the cells are irreversibly damaged and die by apoptosis (http://www.clodronateliposomes.org).

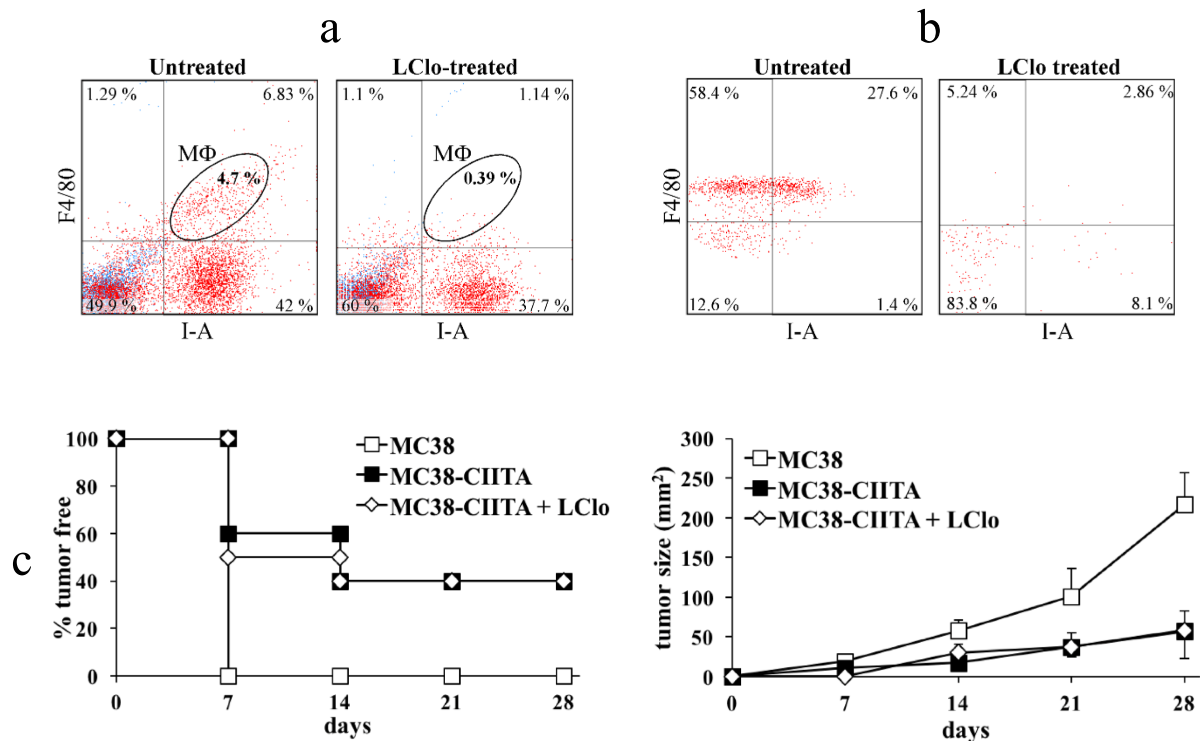



Fig. 11 **MC38-CIITA tumors cells are the only APCs (Macrophages Depletion).** (a) Splenocytes and (b) peritoneal macrophages were taken from mice treated with LClo as described previously and analysed by immunofluorescence co-staining and flow cytometry, for the expression of I-A, and F4/80 using their corresponding directly labelled monoclonal antibodies and compared to untreated mice as a control. (c) Equal number ($5x10^4$) of MC38-CIITA tumor cells were injected in either untreated (MC38-CIITA) or LClo-treated (MC38-CIITA + LClo) CD11c.DTR transgenic mice. A group of untreated mice was also injected with the same number of parental MC38 cells, as control (MC38). Tumor take, measured as percentage of mice with tumors (ordinate, left panel) and kinetics of tumor growth (ordinate, right panel) were followed over time (days in abscissa).

Following the protocol described in Materials and Methods, CD11c.DTR female mice were injected with LClo. To control that the treatment was effective, spleens cells as well as peritoneal macrophages from treated mice were preliminary analyzed for the expression of the F4/80 macrophage marker as well as for the expression of MHC-II molecules present on macrophages also. Results presented in Figure 11 clearly show that a strong depletion of both splenic macrophages (panel a) and peritoneal macrophages (panel b) was obtained after LClo treatment. LClo-treated mice were then injected with MC38-CIITA tumor cells and *in vivo* tumor growth was evaluated over time. 40% of animals were able to reject MC38-CIITA tumor cells after 4 weeks, exactly the same proportion rejecting the tumor cells in control animals not treated with LClo (Fig. 11; c, left panel). Again, even in the case of LClo-treated animals, the kinetics of MC38-CIITA tumor growth in 60% of animals that did not reject the tumor was strongly retarded

as compared to the one of parental tumors and closely followed the kinetics of MC38-CIITA tumors growing in animals that did not undergo LClo treatment (Fig. 11; c, right panel).
Taken together, these results indicate that CIITA-tumor cells can trigger a protective anti-tumor immune response not only in absence of dendritic cells but also in absence of macrophages.

# Part III.
# Chapter 3: Discussion and Conclusion

Our previous studies have demonstrated that CIITA-driven MHC-II gene expression in tumor cells of the H-$2^d$ genetic background can indeed render these cells potent stimulators of a protective and long lasting adaptive anti-tumor immune response capable to protect the syngeneic vaccinated animals against a challenge with MHC-II negative parental tumors and to transfer this protection to naïve recipient by MHC-II-restricted CD4+ TH cells [10], [11], [12], [35]. In the present study we extended this observation to tumor cells of the H-$2^b$ genetic background, which at variance with H-$2^d$ genotype can express only one of the two MHC-II molecules, the I-A but not the I-E molecule. MC38 colon carcinoma tumor cell line, that is MHC-II-negative and highly tumorigenic *in vivo*, became strongly immunogenic after de novo expression of MHC-II molecules mediated by CIITA transfection. Although we could not reach the percentage of full rejection of certain Balb/C tumor cell lines such as the TS/A mammary carcinoma or the WEHI 164 fibrosarcoma [12], [35], up to 50% of animals were able to reject MC38-CIITA tumor cells. Importantly, however, the remaining animals that did not reject the tumor developed an immune response capable to significantly reduce tumor growth, indicating that all animals were indeed responding against the tumor. The reason of absence of full rejection in the second group of mice is most likely related to the possible *in vivo* modulation of MHC-II and MHC-I expression in the injected tumor cells, as we previously observed [10], [35] and confirmed here. Importantly, all rejecting animals were resistant to challenge with parental MHC-II negative tumors and their CD4+ TH cells could transfer protection to naïve H-$2^b$ C57BL/6 mice. These results, definitively establish the general validity of our approach and demonstrate that tumor cells of distinct genetic background and distinct histotypes origin can become immunogenic when expressing CIITA-driven MHC class II molecules. Importantly, the present results indicate that a single MHC-II restricting element, the I-A molecule, could bind sufficient tumor-associated antigens to mediate CD4+ TH cell recognition and triggering of adaptive anti-tumor responses *in vivo.* Although our system is presently confined to an animal approach in which no complete genetic analysis of the tumor mutation rate is available, it is important to stress that the approach works also for tumors like sarcomas with low genetic mutation rate. Thus, we think that this is an advantage over the present human therapies based on antibodies to checkpoint inhibitors in which best results are obtained with tumors of high genetic mutation rate [36], [37].

Although we have previously clearly demonstrated that the MHC-II molecules of tumor cell origin were necessary to mediate the triggering process of the key lymphocytes initiating and maintaining the anti-tumor state, that is the CD4+ TH cells, we could not firmly establish whether the CIITA-tumor cells were indeed themselves the prominent APCs capable to perform *in vivo* priming of naïve TH cells. Indeed, the possibility existed that, upon injection, MHC-II-TAA complexes derived from dying cells or from cell debris could be captured by DCs and used by these professional APCs to trigger and prime naïve CD4+ TH cells. This crucial point was addressed in the present study by the use of the CD11c.DTR transgenic mice, constructed in a C57BL/6 H-2$^b$ background, expressing the diphtheria toxin receptor under the control of the CD11c promoter, mainly expressed in DCs. In these mice, DCs could be conditionally deleted by treatment with diphtheria toxin [15]. Our results clearly demonstrate that MC38-CIITA tumors cells can still be rejected or strongly delayed in their growth in DT-treated CD11c.DTR transgenic mice in a way very similar to that obtained in untreated counterpart or in non-transgenic C57BL/6 mice. Moreover, although it has been reported that DT treatment of CD11c.DTR transgenic mice may also partially ablate macrophages [38], [39], the other important professional APCs *in vivo*, we treated CD11c.DTR transgenic mice with Liposomal Clodronate, a compound that upon selective phagocytosis by macrophages kills the cells by apoptosis [40], and in the spleen ablates particularly the marginal zone macrophages and metallophilic macrophages considered the predominant APCs [41].
Again, upon injection, CIITA-tumor cells could be rejected or strongly retarded in their growth with superimposable behavior as the one observed in liposomal-untreated mice. Thus, CIITA-driven MHC-II positive tumor cells can perform not only antigen processing and presenting function *in vitro* [10], [42] but they can also serve as surrogate APCs *in vivo* to prime naïve CD4+ TH cells and induce potent adaptive immune response against the tumor.
Our results challenge the widely accepted view that antigen priming of naïve tumor-specific CD4+ TH cells *in vivo* can be optimally performed only by professional APCs and particularly by DCs [43]. This view has been supported by the notion that optimal stimulation of naïve TH cells requires two signals, the first provided by the antigen-specific T cell receptor recognizing MHC-II-antigenic peptide complex, and the second provided by the CD28 molecule interacting with co-stimulatory molecules such as the B7.1 (CD80) and B7.2 (CD86) optimally expressed on DCs [44]. Even if tumor cell may express in certain cases MHC-II molecules, they do not express in general costimulatory molecules such as CD80 and CD86 and thus they cannot provide the second signal. MC38 tumor cells used in this study do not express CD80 and CD86 costimulatory molecules and this phenotype is not modified by CIITA transfection (Fig. 2). Yet,

the absence of costimulatory molecules does not prevent CIITA-driven MHC-II expressing tumor to act as potent surrogate APCs *in vivo*. Thus, either CIITA-tumors do not need accessory molecules to perform their APCs function *in vivo,* or other accessory molecules are involved to provide the second signal. Future investigation will be focused on this important issue.

Another important consideration that stems from our studies relates to the anatomical location where CIITA-tumor cells' APC function takes place. It is widely accepted that priming of antigen-specific naïve CD4+ T cells by professional APCs takes place in secondary lymphoid organs such as lymph nodes and spleen. There, DCs that have captured and processed the antigens in the periphery migrate and present antigenic peptides within the context of MHC-II molecules [45]. However, previous studies of our group have shown that, in contrast to parental tumors where leukocyte infiltrate is scanty, CIITA-tumors rapidly become infiltrated first by CD4+ T cells and subsequently by other cells such as DCs, CD8+ T cells and macrophages [10], thus reorienting the tumor microenvironment from a pro-tumor to an anti-tumor microenvironment [5]. Moreover, it has been shown that lymphocytes can organize themselves in lymphoid tissues different from classical lymph nodes, the so-called tertiary lymphoid organs or ectopic lymphoid-like structures, peculiar lymphoid formations found in inflamed and, interestingly, in tumoral tissues [46]. They show many characteristics of lymph nodes associated with the generation of an adaptive immune response [47]. Thus it is tempting to speculate that tumor cells endowed with CIITA-driven MHC-II expression not only may exert full APCs function for priming naïve tumor-specific CD4+ T cells but may also perform this activity within the tumor tissue itself where a rudiment of organized lymphoid structure can be generated.

Finally, considering the high *in vivo* immunogenicity of CIITA-driven MHC-II expressing tumors it is clear that these tumor cells optimally express the relevant MHC-II-TAA complexes and key neoantigens for immune stimulation. These cells therefore may be instrumental to purify and sequence the key TAA that in conjunction with similarly derived HLA class I-restricted TAA peptides, will be the basis for the construction of a multi-peptide, multi-epitope vaccine that can target both CD4+TH and CD8+ CTL anti-tumor responses.

# Abbreviations List

ACT: Adoptive Cell Transfer

APC: Antigen Presenting Cell

BCR: B Cell Receptor

βm: Beta 2 Microglobulin

CD: Cluster of Differentiation

CD40L: CD40 Ligand

CIITA: Class II Transactivator

CLIP: Class II-associated Invariant chain Peptide

CREB: Cyclic AMP Response Element Binding protein

CTL: Cytotoxic T Lymphocytes

DC: Dendritic Cell

DT: Diphtheria Toxin

DTR: Diphtheria Toxin Receptor

ER: Endoplasmic Reticulum

Fc: Fragment Crystallizable

FCS: Fetal Calf Serum

HAT: Histone Acetyl-Transferase

HLA: Human Leukocytes Antigens

i.p.: intraperitoneally

IFN-γ: Interferon-γ

Ig: Immunoglobulin

IL: Interleukin

LClo: Liposomal Clodronate

m.f.i.: Mean Fluorescence Intensity

MHC: Major Histocompatibility Complexe

MHC-I/II: Major Histocompatibility Complexe Class I/II

NF-Y: Nuclear Factor Y

OVA: Ovalbumin

RFX: Regulatory Factor X

s.c.: subcutaneously

TAA: Tumor Associated Antigen

TAP: Transporter associated with Antigen Processing

TCR: T Cell Receptor

TGF-β: Tumor Growth Factor-β

Th: T helper

Treg: Regulatory T cells

TS/A: Mammary Adenocarcinoma

WT: Wild Type

## Schemes and Figures Lists

**Scheme 1** The main steps of the humoral immune response ........ 11

**Scheme 2** Mechanism of CTL mediated toxicity ........ 13

**Scheme 3** Amplification of CD8+ T cell response by CD4+ T cell ........ 14

**Scheme 4** Priming of naive CD4+ T cells by 3 signals provided by APCs ........ 15

**Scheme 5** The different subsets of CD4+ effector T cells and the molecules that they secrete or express ........ 16

**Scheme 6** Gene structure of human and mouse MHC ........ 18

**Scheme 7** The structure of MHC class I molecule ........ 19

**Scheme 8** Pathways of antigen processing and presentation ........ 20

**Scheme 9** Structure of a class II MHC molecule ........ 21

**Scheme 10** Mechanism of Action of CIITA ........ 23

**Scheme 11** The three Es of cancer immunoediting ........ 25

**Scheme 12** Tumor Cell as Antigen Presenting Cells ........ 26

Fig. 1 **MHC-II positive MC38-CIITA clone generation** ........ 32

Fig. 2 **Lack of the expression of CD80 and CD86 costimulatory molecules on both parental and CIITA transfected tumor cells** ........ 33

Fig. 3 **No effect for CIITA gene expression on the proliferation rate of MC38-CIITA** ........ 34

Fig. 4 **MC38-CIITA tumor cells are rejected or drastically retarded in their growth in vivo.** ........ 35

Fig. 5 **Vaccination with MC38-CIITA induces long lasting immunity against MC38 parental tumor** ........ 36

FIG. 6 **LOSS OF MHC-I AND LOW LEVEL OF MHC-II EXPRESSION ON THE EX VIVO MC38-CIITA TUMORS.** .......... 37

FIG. 7 **IMMUNE ACTIVATED SPLENOCYTES FROM MC38-CIITA VACCINATED MICE SECRETE HIGH LEVEL OF IFN-G** .......... 38

FIG. 8 **CD4+ TH CELLS ARE THE KEY CELLS RESPONSIBLE FOR THE ANTI-TUMOR IMMUNITY INDUCED AFTER MC38-CIITA VACCINATION** .......... 39

FIG. 9 **CD11C.DTR MICE GENOTYPING** .......... 40

FIG. 10 **MC38-CIITA TUMORS CELLS ARE THE ONLY APCS (DCS DEPLETION)** .......... 41

FIG. 11 **MC38-CIITA TUMORS CELLS ARE THE ONLY APCS (MACROPHAGES DEPLETION)** .......... 42

# Publications

1. **Farah Bou Nasser Eddine,** Greta Forlani, Letizia Lombardo, Alessandra Tedeschi, Giovanna Tosi & Roberto S. Accolla,
   "CIITA-driven MHC class II expressing tumor cells can efficiently prime naive CD4+ TH cells *in vivo* and vaccinate the host against parental MHC-II- negative tumor cells"
   *ONCOIMMUNOLOGY,* Vol. 6, no. 1 Jan 2017

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