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New anti-tumor vaccine and immunotherapeutic strategies based on optimal stimulation of $CD4^+$ T Helper cells.

Thesis of

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Ph.D. Program "Experimental Medicine and Oncology" - XXII Cycle

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Dedicato a Mamma e Papà, Fabio, Leo e… Maurizio,

senza i quali nulla di tutto ciò sarebbe stato così importante…

"Ad Augusta per Angusta"

Parte seconda

ABSTRACT

Vaccination with a poorly or not immunogenic tumor fails to protect the host from a subsequent challenge with the same tumor. The mechanisms underlying the failure of these tumors to sensitize therapeutic T cells are not clearly understood, but the inability of host T cells to recognize tumor has been implicated. Previous works from our laboratory have shown that the highly aggressive BALB/c mammary adenocarcinoma TS/A, can be rejected if genetically modified to express MHC class II genes upon transfection of the MHC class II transactivator CIITA. MHC class II molecules are required for presenting antigenic peptides to T helper cell and thus activate the cascade of events leading to immune effector functions such as antibody production and cytolytic T cell activity.

The purpose of my thesis was to investigate whether the above approach could be generalized and thus extended to tumors of distinct histological origin. Moreover, it was assessed whether anti-tumor lymphocytes generated by this approach could be used as an immunotherapeutic tool for established cancers.

Beside the previously described TS/A-CIITA breast carcinoma cells, stable CIITA-transfectants of colon adenocarcinoma C51, renal adenocarcinoma RENCA, and sarcoma WEHI-164, were generated. Tumor cells transfectants were injected *in vivo* and their grow kinetics and recipient's immune response were analyzed. Tumor rejection and/or retardation of growth was found for CIITA-transfected C51 and RENCA adenocarcinomas, as well as WEHI-164 sarcoma. As for TS/A, this tumor rejection correlated mostly with the stability and the amount of CIITA-induced MHC class II expression. Interestingly, mice rejecting CIITA-

transfected tumors acquired specific immunological memory, as demonstrated by resistance to challenge with untransfected parental tumors. Adoptive cell transfer experiments demonstrated that tumor immunity correlates with $CD4^+$ and $CD8^+$ T lymphocytes.

In this thesis, it is also shown that CIITA-induced MHC class II expression on tumor cells is able to generate antitumor therapeutic T cells. Indeed, T cells from TS/A-vaccinated mice were used in an adoptive cell immunotherapy (ACT) model of established tumors. The results showed the cure at early stages and a significantly prolonged survival at later stages of tumor progression. Importantly, $CD4^+$ T cells were clearly superior to $CD8^+$ T cells in anti-tumor protective function. Interestingly, the protective phenotype was associated to both a Th1 and Th2 polarization of the immune effectors.

Of great importance, the T cells obtained from vaccinated mice were therapeutic also without other *in vitro* activation passages, like treatment with anti-CD3 and IL-2, differently to some other approaches of vaccination and ACT. Furthermore, ACT induced proliferation of tumor-specific immune splenocytes in receiving mice, leading to rejection of subsequent tumor challenge or to protection from metastasis in animals having established tumors.

These data support our original idea that CIITA-expressing tumor cells can act as antigen-presenting cells (APCs) for their own tumor-associated antigens to $CD4^+$ T cells, to induce specific and potent anti-tumor responses. These results establish the general application of our tumor vaccine model.

This approach let us to envisage additional applications of this strategy for producing better lymphocyte effectors for adoptive anti-tumor cell immunotherapy, particularly in those cases of tumors non-responsive to existing therapies.

ABBREVIATIONS

Ab	Antibody
APC	Antigen Presenting Cell
ACT	Adoptive Cell Therapy
CBP	CREB Binding Protein
CIITA	Class II Transactivator
CREB	Cyclic AMP Response Element Binding protein
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
ER	Endoplasmic Reticulum
HAT	Histone Acetyl-Transferase
HLA	Human Leukocyte Antigens
IFN-γ	Interferon-γ
Ii	Invariant Chain
IL	Interleukin
MHC	Major Hystocompatibility Complex
MIIC	MHC class II-containing compartment
NK	Natural Killer cell
NF-Y	Nuclear Factor Y
pCAF	p300/CBP-Associated Factor
RFX	Regulatory Factor X
s.c.	Subcutaneously
SFC	Spot-forming Cell
SRC-1	Steroid Receptor Co-activator 1
TAA	Tumor Associated Antigen
TAP	Transporter Associated with Antigen Processing
TCR	T cell Receptor
TGF-β	Transforming Growth Factor-Beta
Th1	T helper cell type 1
Th2	T helper cell type 2
TIL	Tumor Infiltrating Lymphocyte
TSA	Tumor Specific Antigen
TS/A	Mammary adenocarcinoma cells
TS/A pc	Mammary adenocarcinoma parental cells
VEGF	Vascular Endothelial Growth Factor

SCIENTIFIC PUBLICATIONS

1. Bossi E, Soragna A, Miszner A, Giovannardi S, ***Frangione V***, Peres A. Oligomeric structure of the neutral amino acid transporters KAAT1 and CAATCH1. Am J Physiol Cell Physiol. 2007; 292(4):C1379-87.

2. Mortara L, ***Frangione V***, Castellani P, De Lerma Barbaro A, Accolla RS. Irradiated CIITA-positive mammary adenocarcinoma cells act as a potent anti-tumor-preventive vaccine by inducing tumor-specific $CD4^+$ T cell priming and $CD8^+$ T cell effector functions. Int Immunol 2009;21:655-65.

3. ***Frangione V***, Mortara L, Castellani P, De Lerma Barbaro A, Accolla RS. CIITA-driven MHC-II positive tumor cells: preventive vaccines and superior generators of anti-tumor $CD4^+$ T lymphocytes for immunotherapy. Int. J. Cancer , 2010, in press.

ABSTRACTS

1. Bossi E, Soragna A, Giovannardi S, ***Frangione V***, Miszner A, Peres A. "Oligomerization and independent functioning of the two neutral aminoacid transporters CAATCH1 and KAAT1". Joint Meeting of The German Society of Physiology and The Federation of European Physiological Societies – Munich – GERMANY, March 2006.

2. Giovannardi S, ***Frangione V***, Bossi E, Miszner A, Soragna A, Peres A. "Homo- and heterooligomerization between some members of the Na^+/Cl^- - dependent transporter family, KAAT1, CAATCH1 and rGAT1, seen as FRET". Meeting "Transporters 2006" – Parma – ITALY, September 2006.

3. Miszner A, Bossi E, ***Frangione V***, Peres A. "Single amino acid substitution changes substrate selectivity of the Manduca sexta neutral amino acid transporter KAAT1". Meeting "Transporters 2006" – Parma – ITALY, September 2006.

4. ***Frangione V***, Mortara L, Accolla RS. "Tumor rejection and anti-tumor memory to neoplasias of different histotype genetically modified to express CIITA-driven autologous MHC class II genes. A new strategy of anti-tumor vaccination and tumor immunotherapy". 6th National Conference SIICA – Italian Society of Immunology, Clinical Immunology and Allergology – Rome – ITALY, June 2008.

5. Pellegrini F, Marinoni M, Mortara L, ***Frangione V***, Accolla RS, Nespoli L. "Phenotypic and functional study of the regulatory T-lymphocyte population ($CD4^+CD25^{high}FoxP3^+$) in in patients with Down syndrome". 64° National Conference Italian Society of Pediatrics – Genoa – ITALY, October 2008.

6. ***Frangione V***, Mortara L, De Lerma Barbaro A, Accolla RS. "The immunological basis of tumor rejection and antitumor memory to neoplasias of different histotypes genetically modified to express CIITA-driven autologous MHC class II genes". Annual meeting of the French Society for Immunology – Paris – France, November 2008.

7. Mortara L, ***Frangione V***, Castellani P, Pinter M, De Lerma Barbaro A, Accolla RS. "Irradiated CIITA-positive mammary adenocarcinoma cells act as effective tumor cell vaccine inducing tumor-specific $CD4^+$ and $CD8^+$ T cell immune responses and homologous tumor rejection capacity". Annual meeting of the French Society for Immunology – Paris – France, November 2008.

8. ***V. Frangione***, L. Mortara, P. Castellani, A. De Lerma Barbaro, R.S. Accolla. Tumor rejection and anti-tumor memory to cancer cells of different histotype genetically modified to express CIITA-driven autologous MHC class II genes. A new strategy of anti-tumor vaccination and

tumor immunotherapy. 2nd European Congress of Immunology (EFIS), Berlin, Germany, September 13-16, 2009.

9. L. Mortara, ***V. Frangione***, P. Castellani, A. De Lerma Barbaro, R.S. Accolla. Irradiated CIITA-positive mammary adenocarcinoma cells act as a potent antitumor preventive vaccine by inducing tumor-specific CD4+ T cell priming and CD8+ T cell effector functions. 2nd European Congress of Immunology (EFIS), Berlin, Germany, September 13-16, 2009.
10. L. Mortara, ***V. Frangione***, P. Castellani, A. De Lerma Barbaro, R.S. Accolla. Non-replicating MHC Class II- CIITA-positive mammary adenocarcinoma cells act as an effective anti-tumor-preventive vaccine by inducing long-lasting tumor-specific CD8+ T cells and potent CD4+ T helper cells. 51th Annual Meeting of the Italian Cancer Society (SIC), Milan, Italy, November 23-26, 2009.

MEETINGS AND CONFERENCES

1. Joint Meeting of The German Society of Physiology and The Federation of European Physiological Societies – Munich – GERMANY, March 2006.
2. Meeting “Transporters 2006” – Parma – ITALY, September 2006.
3. 6th National Conference SIICA – Italian Society of Immunology, Clinical Immunology and Allergology – Rome – ITALY, June 2008. ***Frangione V. oral presentation "Workshop – Tumor Immunology"***

TABLE OF CONTENT

1. INTRODUCTION

1.1. The Immune System

The immune system is a network of molecules, cells and organs that has evolved to recognize foreign substances in the body and destroy them. The targets of the immune defences are pathogens, such as viruses, bacteria, fungi, and other parasites.

At the heart of the immune response is the ability to distinguish between "*self*" and "*non-self*". Every cell in the body carries distinctive molecules that distinguish it as *"self"*. Normally the body's defences do not attack tissues that carry a self-marker; rather, immune cells coexist peacefully with other cells in a state known as *"self-tolerance"*. Most foreign molecules carry distinctive markers. The immune system is able to recognize many millions of distinctive non-self molecules, and to respond in different ways. An *"immunogenic antigen"* is a substance capable of triggering an immune response (e.g., bacterium, virus, cells from another individual).

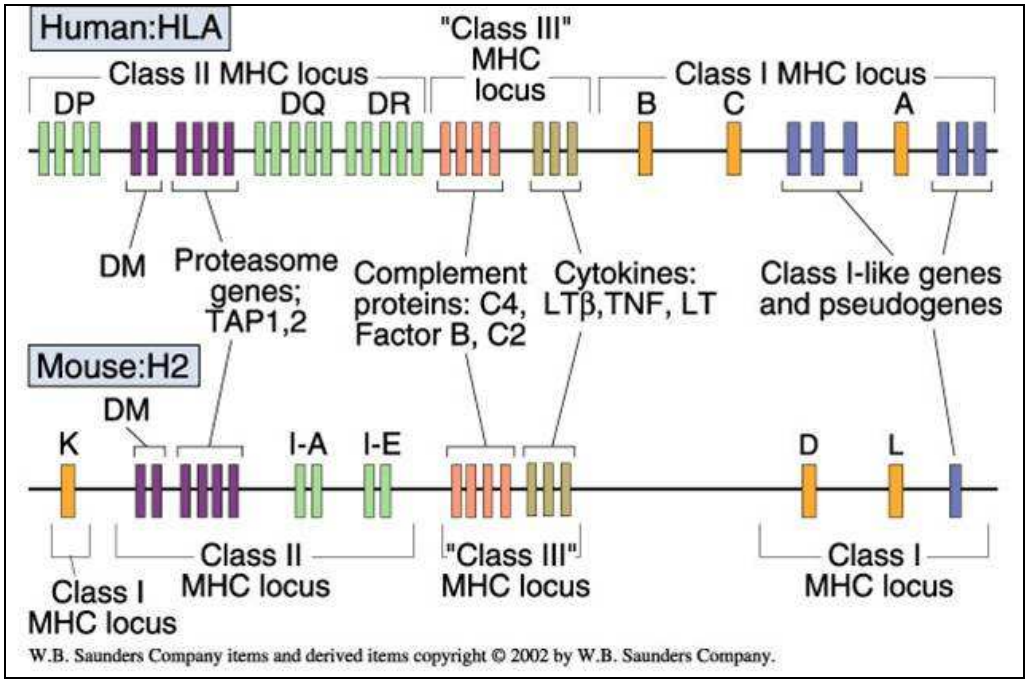
The architecture of the immune system is multi-layered, with defences on several levels. Once pathogens have entered the body, they are dealt with by the *"innate immune system"* and by the *"acquired or adaptive immune system"*. Both systems consist of a multitude of cells and molecules that interact in a complex manner to detect and eliminate pathogens.

The innate system represents the first line of defence to an intruding pathogen. The response evolved is therefore rapid, and is unable to "*memorise*" the same said pathogen should the body be exposed to it in the future. Although the cells and molecules of the adaptive system possess slower temporal dynamics, they possess a high degree of specificity and evoke a more

potent response on secondary exposure to the pathogen indicating the acquisition of specific memory.

1.2. Major Histocompatibility Complex and Antigen Presentation

The Major Histocompatibility Complex (MHC) is a large cluster of genes found on the short arm of chromosome 6 in humans (HLA; human leukocyte antigens) and on chromosome 17 in mice (H2) (***Scheme 1***).



Scheme 1. Schematic overview of the genes of the MHC locus (From: *Abbas and Lichtman, 2003*).

The MHC genes and their products are grouped into two classes because of their chemical structure and biological properties. Class I molecules are made up of one heavy chain (45 kD) encoded within the MHC and a light chain, called β2-microglobulin (β2M-12 kD) encoded by

a different chromosome. Class II molecules consist of two (α and β) chains of similar size (34 and 30 kD) and both encoded within the MHC.

The products of the MHC play a fundamental role in regulating immune responses. T cells must recognise antigen as a complex with MHC molecules. This requires antigen to be processed by unfolding and proteolytic digestion before it complexes with the MHC molecule. Once formed the complex of antigenic peptide and MHC are generally very stable (half-life ~ 24hrs). Thus, the biological role of MHC proteins is to bind small peptides and to *"present"* these at the cell surface for the inspection of T cell antigen receptors. The MHC is highly polymorphic and the allelic variation of MHC molecules is functionally reflected: each allelic product has a unique set of peptides, which it can bind with high affinity.

The two classes of MHC molecule are specialised to present different sources of antigen. MHC class I molecules present endogenously synthesised antigens, e.g. viral proteins. MHC class II molecules present exogenously derived proteins, e.g. bacterial products or viral capsid proteins. The cell biology and expression patterns of each class of MHC are tailored to meet these distinct roles (*Table 1*).

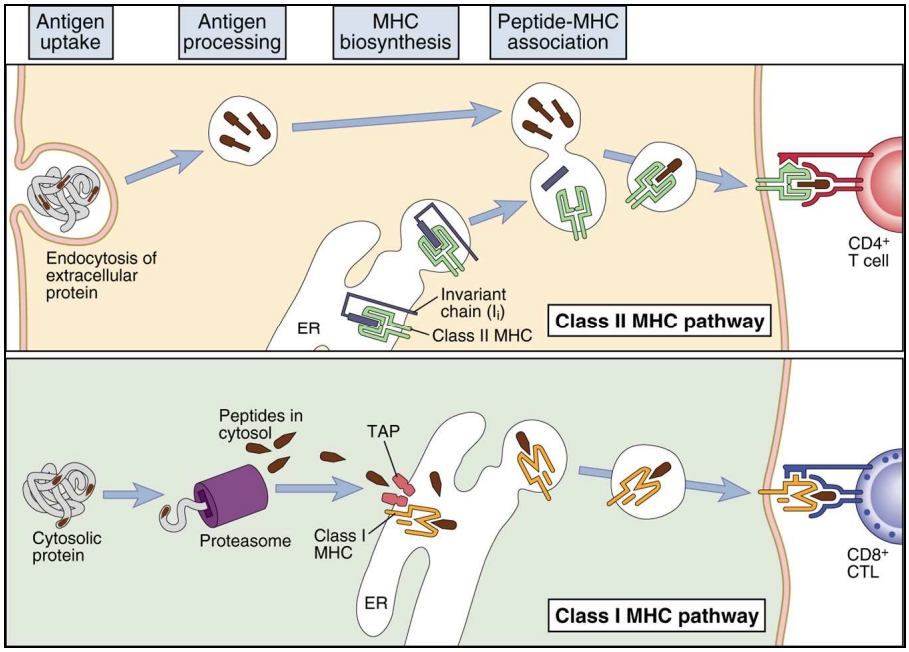
CELL TYPE	MHC I	MHC II
T cells	+++	Varies, inducible in some species
B cells	+++	++
Macrophages	+++	+
Dendritic cells	+++ (x10)	+++ (x10)
Granulocytes	++	-
Endothelium	++	- (inducible)
Hepatocytes	+	-

Table 1.Expression of MHC class I and MHC class II molecules on immune cells.

MHC class I molecules are widely expressed, though the level varies between different cell types, and their function is to present fragments of proteins that are synthesised inside the cell (endogenous). The peptide antigens presented in this manner are checked by killer T-cells, which have receptors for the class I MHC proteins. The purpose of this surveillance system is to identify abnormal body cells, such as those infected with viruses or those that have turned malignant. Such cells will display unfamiliar peptide antigens, e.g. fragments of viral proteins, and are attacked and destroyed. MHC class I molecules are very unstable in the absence of peptide. They bind peptides in the endoplasmic reticulum (ER). Peptides are generated continuously in the cytoplasm by the degradation of proteins, predominantly by the proteasome. Peptides of suitable length (~8-18 amino acids) are specifically transported across the ER membrane by a heterodimeric transporter made up of the Transporter Associated with Antigen Processing (TAP) molecules. These peptides bind to the class I MHC molecules in the ER if they have appropriate sequence motifs, the MHC molecules being stabilised in a partially folded state by chaperone proteins. The peptide bound class I MHC molecules adopt their fully folded conformation, release chaperone proteins and transit to the cell surface via the Golgi (***Scheme 2***).

MHC class II molecules are constitutively expressed only by certain cells involved in immune responses, although they can be induced on a wider variety of cells. The immune cells expressing MHC class II molecules are specially designed to present peptide antigens derived from exogenous digested particles. The antigens are presented to helper T-cells (Th), which have receptors for MHC class II proteins. Upon recognition of MHC class II-bound peptides, Th cells are triggered and can subsequently activate the cascade of events leading to immune effector functions such as antibody production and cytolytic T cell activity. During the intracellular processing, MHC class II molecules bind to a third polypeptide in the ER, called

invariant chain or Ii. The invariant chain serves two purposes. It blocks the binding of peptides to the class II molecule and it targets the class II molecule to a specialised endosomal compartment, the MHC class II-containing compartment (MIIC). Exogenous antigens enter the cell in membrane vesicles, either by fluid phase pinocytosis or by receptor-mediated endocytosis. These vesicles fuse with the MIIC compartment. The MIIC compartment has an acid pH and contains proteases; this combination unfolds and degrades both the antigen and the invariant chain causing the generation of antigenic peptides and the release of class II molecules to bind those peptides with appropriate sequence motifs. Then, the class II molecules, peptide-complexed or *"empty"*, traffic to the plasma membrane (***Scheme 2***).



Scheme 2. Schematic overview of MHC class I and MHC class II (right) intracellular processing and antigen presentation pathways (From: *Abbas and Lichtman, 2003*).

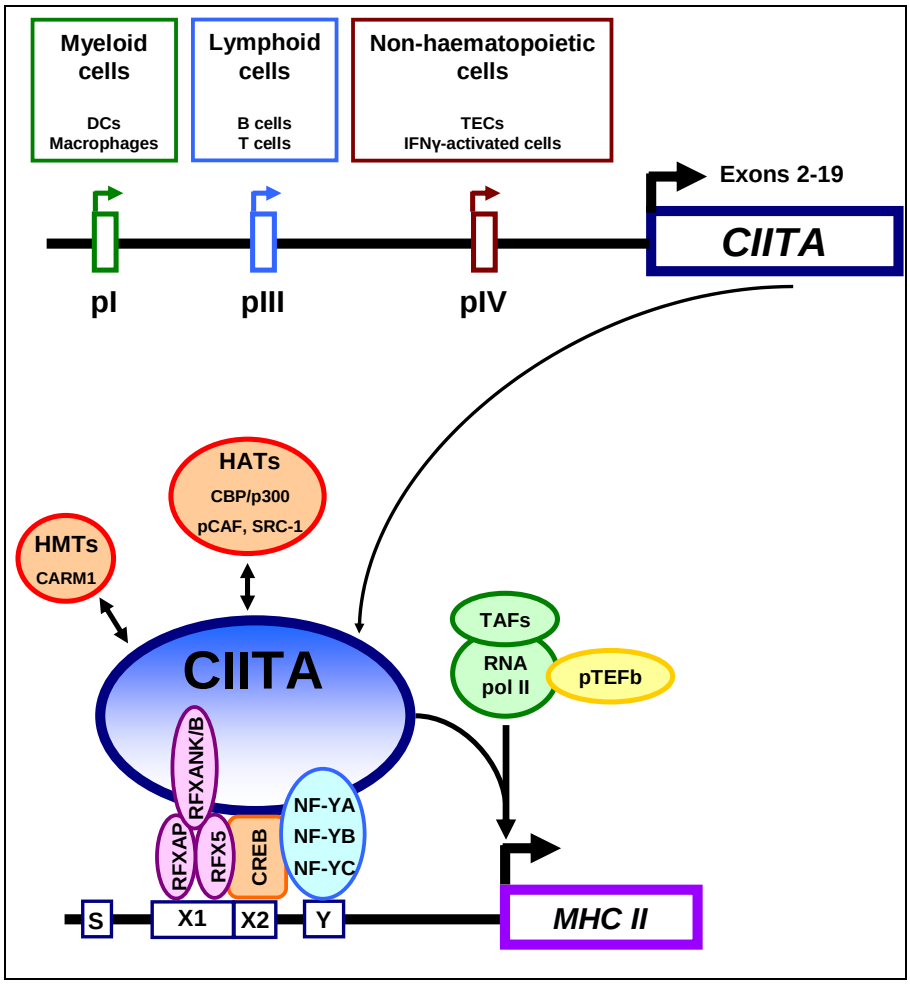
1.3. CIITA and MHC class II molecules

The class II transactivator (CIITA) is the master regulator of MHC class II genes (*Chang et al. 1994; Chang et al. 1996*). CIITA is a transcriptional co-activator that does not bind DNA, but interacts with DNA-binding proteins called RFX (regulatory factor X; consisting of RFX5/RFXANK-RFXB/RFXAP), CREB (cyclic AMP response element binding protein), and NF-Y (nuclear factor Y; consisting of NF-YA, NF-YB, and NF-YC) (*Kern et al. 1995; Zhu et al. 2000*). All these transcription factors directly bind to the X and Y conserved *cis*-acting sequence motifs of the MHC class II promoters, and they interact with distinct or overlapping domains of CIITA (***Scheme 3***).

CIITA also activates and regulates the transcription of genes encoding MHC class II through the recruitment of histone acetyl-transferases (HATs), including CREB-binding protein (CBP) (*Kwok et al. 1994*), p300, p300/CBP-associated factor (pCAF) and steroid receptor co-activator (SRC)-1, which can acetylate histone at lysine residues to allow gene activation (*Zika and Ting, 2005*). Thus, CIITA appears to be a focal point of interaction for both DNA-binding proteins specific for the MHC class II promoters and for HATs to allow chromatin opening (*Wright and Ting, 2006*) (***Scheme 3***).

The MHC class II transactivator was initially identified and characterized by a somatic cell genetic approach. After the generation of a somatic cell mutant (Rj 2.2.5), negative for MHC-II gene expression (*Accolla et al. 1983*), somatic complementation with murine MHC class II positive cells demonstrated the existence of a dominant locus, encoding the *trans*-acting activator. This locus was mapped to mouse chromosome 16 and designated *Air-1* (*Accolla et al. J.Exp.Med. 1985; Accolla et al, PNAS 1985; Accolla et al. 1986*). Several years later, the

product of the human equivalent of the *Air-1* locus (AIR-1) was cloned by a gene complementation approach and designated CIITA (*Steimle et al. 1993*).



Scheme 3. The promoter of MHC class II gene.

CIITA is absolutely required for the constitutive expression of MHC class II in B cells and dendritic cells and for the cytokine induction of these genes in a variety of other cell types. Gamma interferon (IFN-γ) is a prime example of a cytokine, which induces CIITA and subsequently MHC class II expression (*Steimle et al. 1994*).

Two of its promoters, promoter III and promoter IV, are responsible for IFN-γ inducibility (*Muhlethaler-Mottet et al. 1998; Piskurich et al. 1998; Piskurich et al. 1999*). Promoter III contains

a proximal sequence that is responsible for the constitutive expression of CIITA in B cells and a distal sequence, which confers IFN-γ responsiveness. Promoter IV contains the major IFN-responsive sequence (***Scheme 3***).

1.4. Adaptive Immunity

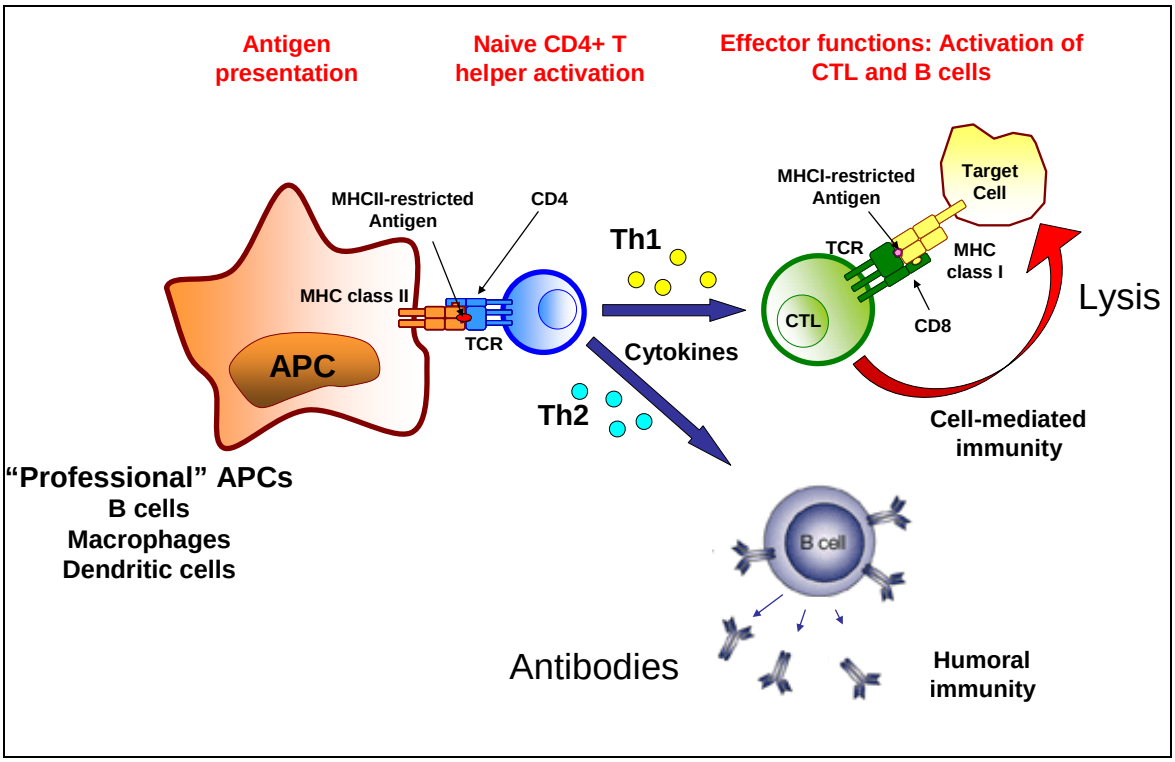
The adaptive immune system is so-called because it adapts or *"learns"* to recognize specific antigens, and retains a *"memory"* of them for speeding up future responses. The learning occurs during a primary response to a kind of antigen not encountered before by the immune system. After the ending of primary response against the antigen, the immune system retains a *"memory"* of the kind of antigen that caused the reaction and this immune memory can confer protection up to the lifetime of the organism.

Lymphocytes are the principal active components of the adaptive immune system. The other components are antigen-presenting cells (APCs), which trap antigens and bring them to the attention of lymphocytes so that they can mount their attack.

Adaptive immune responses are initiated in the lymphoid tissue, in response to antigens presented by *"professional antigen-presenting cells"*, which role is to activate antigen-specific naive T lymphocytes (***Scheme 4***).

The differentiation of naive $CD4^+$ T cells into distinct classes of $CD4^+$ effector T cells - Th1 or Th2, for example - depends on the effects of the cytokines present during the initial phase of $CD4^+$ T-cell activation.

Antigen-specific effector T cells and antibody-secreting B cells are generated by clonal expansion and differentiation over several days.



Scheme 4. Adaptive Immune Response.

The effector phase of adaptive immune responses involves the clearance of extracellular infectious particles by antibodies (*humoral immunity*) and the clearance of intracellular residues of infection through the actions of effector CTLs, which lyse infected cells.

In particular, Th1 response is generally involved in helping to activate the $CD8^+$ cytotoxic T cells that will recognize infected cells and destroy them. Naive $CD4^+$ T cells initially stimulated in the presence of IFN-γ tend to develop into Th1 cells. In part, this is because this cytokine induces or activates the transcription factors leading to Th1 development, and in part because IFN-γ inhibits the proliferation of Th2 cells. Th2 response, instead, is generally involved in the activation of B cells that will produce antibodies. Naive $CD4^+$ T cells activated in the presence of IL-4 tend to differentiate into Th2 cells.

1.5 Tumor Immunology

Tumors are a major health problem worldwide and one of the most important causes of morbidity and mortality. Tumors arise from the uncontrolled proliferation and spread of clones of transformed cells. One of the most controversial questions in immunology for over a century has been whether the immune system could recognize and eliminate malignant tumors.

The immune system coevolved with infectious diseases in a way that allows survival of the host and the pathogen. In contrast, because tumors usually arise in individuals beyond reproductive age, they are more a problem for the individual patient than for the population as a whole. Thus, the immune system did not develop special strategies to protect against cancer; instead, tumors are fought by an immune system that has been shaped by infectious pathogens. Indeed, the immunobiology of cancer and infectious diseases may be overlapping, *e.g.*, the recognition and elimination of both virus-infected and tumor cells seem to depend on similar mechanisms.

From an immunologic perspective, tumor cells can be viewed as altered self-cells that have escaped normal growth-regulating mechanisms. In 1950, Macfarlane Burnet postulated the hypothesis of *"immune surveillance"* for which a physiologic function of the immune system is to recognize and destroy clones of transformed cells before they grow into tumours and to kill tumours after they are formed. Burnet proposed that the immune system defines the *"self"* before birth, and antigens, which make their first appearance after the immune system has reached maturity, would automatically be regarded as *"non-self"* and attacked (*Burnet , 1970*).

A critical feature of the immune surveillance hypothesis is that the default reaction of the mature immune system to new antigens is activation, and so the major question posed to

cancer researchers by this hypothesis was how tumours managed to sneak through this surveillance mechanism.

The tumour cells have developed a process of *"immune evasion"* often called *"tumour escape"* which may be a result of several mechanisms (*Finke et al. 1999; Dunn et al. 2004*).

Downregulation of MHC class I expression

Malignant transformation of cells is often associated with a reduction (or even a complete loss) of MHC class I molecules and the selection of MHC class I-deficient tumor escape variants is an important phenomenon frequently seen in human tumors (*Festenstein and Garrido, 1986; Garrido et al. 1997; Carretero et al. 2008*).

Since $CD8^+$ CTL recognize only antigen associated with MHC class I molecules, any alteration in the expression of these molecules on tumour cells may exert a profound effect on CTL-mediated immune response.

Within this frame, tumor cells behave similarly to cells infected by certain viruses. Indeed, viruses often escape CTL recognition by using their own products to reduce MHC class I expression to the cell surface. This aim can be reached in several ways, from the inhibition of class I transcription, the inhibition of assembly of class I heavy chain to beta-2 microglobulin, the inhibition of transport of the mature MHC-I heterodimer to the cell surface, etc. (*Ploegh, 1998; Yewdell and Bennink, 1999*).

Moreover, tumor-derived interleukin (IL)-10 can lead to reductions in MHC molecules (*de Waal-Malefyt et al. 1991; Matsuda et al. 1994*) and TAP expression (*Salazar-Onfray et al. 1997*).

Modulation of tumour antigens

Certain tumour-specific antigens (TSAs) have been observed to disappear from the surface of tumour cells (*Jager et al. 2001*). Such *"antigen loss variants"* are common in rapidly growing tumours (*Maeurer et al. 1996*). It has also been demonstrated that clonally expanded tumour cells are genetically unstable and can readily acquire new mutations or downregulate tumour suppressor genes through epigenetic mechanisms (*Hanahan and Weinberg, 2000; Vogelstein and Kinzler, 2004*).

Loss of signaling molecules

In cancer patients and in some tumour-bearing mice, alterations in signal transduction molecules are observed in T cells and natural killer (NK) cells (*Whiteside, 1999; Lai et al. 1996; Levey and Srivastava, 1996*).

The cytoplasmic domain of the CD3 ζ subunit in the T cell receptor (TCR) complex, for example, is involved in signal transduction and a decrease in its levels has been reported in T cells from tumor-bearing mice (*Mizoguchi et al. 1992*). Furthermore, alterations or decreased expression of these molecules were also shown in lymphocytes from colon cancer patients (*Nakagomi et al. 1993; Matsuda et al. 1995*) or in patients with renal cell carcinoma (*Tartour et al. 1995*) and melanoma (*Zea et al. 1995*).

These changes, although not antigen-specific, appear to start at the site of the tumour and eventually become detectable in peripheral blood T cells or splenocytes, suggesting that tumour microenvironment induces alterations in the signal transduction pathways. These changes in signalling molecules can often be related to *"immune dysfunction"* observed in the patients.

Products of tumour cells suppress anti-tumour immune responses

A variety of tumor-derived factors contribute to the emergence of complex local and regional immunosuppressive networks, including vascular endothelial growth factor (VEGF), IL-10, transforming growth factor-beta (TGF-β), soluble Fas and Fas ligand.

IL-10 and TGF-β, for example, can be produced by the tumor cells themselves or by infiltrating stromal cells (*Kruger-Krasagakes et al. 1994*).

IL-10 has been shown to block dendritic cell-mediated priming of T cells into CTL effectors *in vitro* (*Steinbrink et al. 1999*), and its expression in serum appears to have negative prognostic impact in certain cancers (*Wittke et al. 1999*).

TGF-β, an immunosuppressive cytokine, inhibits the proliferation and effector functions of lymphocytes and macrophages. TGF-β, indeed, has been shown to be sufficient to drive *in situ* expansion of regulatory T cells, thus bringing together these two evasion mechanisms (*Peng et al. 2004*). TGF-β acts also on non-transformed cells, present in the tumor mass, as well as distal cells in the host to suppress antitumor immune responses creating an environment of immune tolerance, augmenting angiogenesis, invasion and metastasis, and increasing tumor extracellular matrix deposition (*Moutsopoulos et al. 2008*).

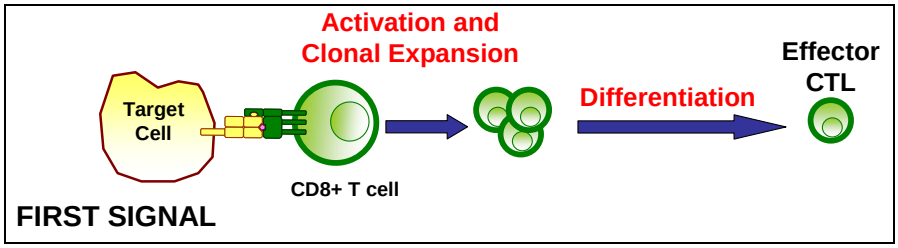
An additional escape mechanism adopted by tumour cells is expression of Fas ligand (FasL) by some tumours, which can induce apoptosis of tumor-infiltrating lymphocytes (TILs) (*Hahne et al. 1996; Song et al. 2001*).

1.6 Cancer Vaccination and Immunotherapy: Different Strategies

The critical goal for immune–based approaches against cancer is from one side to create strategies of vaccination for preventing cancer onset and from the other side to set up strategies of immunotherapy, which can provide long-term and specific eradication of tumor cells (*Berzofsky et al. 2004*).

The ability to discriminate tumor cells from normal tissues is the first step for enabling effective tumor destruction while minimizing toxicity (***Scheme 5***).

Most non hematopoietic tumors express MHC class I molecules, but do not express MHC class II molecules, therefore it is generally considered that the predominant tumoricidal effector mechanism is killing by $CD8^+$ T cells. Most antitumor approaches were thus focussed on the possibility to trigger these effector cells.



Scheme 5. Activation of specific effector CTLs by target cells, the first signal.

1.6.1. Vaccines

Since the discovery of tumor-associated antigens during the early 1990s, rapid progress has been made in identifying antigens and describing immune interactions in cancer patients (*van der Bruggen et al. 1991; Boon et al. 1996; Boon et al. 2006*). The cancer vaccine approach, in particular, delineates the use of active immunizations, designed to stimulate the adaptive arm

of the immune system directly *in vivo* and to induce large numbers of antigen-specific T cell populations with effector functions that are able to mediate immune protection against growing tumors.

The idea of implement vaccines against cancer derived probably from the widespread success for the vaccines in the field of viral diseases prevention, that provided a considerable base of immunologic information on the mechanisms underlining the preventive responses and are easily administered to outpatients and generally do not cause significant side effects.

In contrast to viruses and other pathogens, however, vaccines containing recombinant proteins or synthetic antigenic peptides usually fail to induce significant immune responses. During the past years, different cancer vaccines based on synthetic peptides, 'naked' DNA, dendritic cells, recombinant viruses and recombinant adenoviruses have been clinically tested. However, using conventional oncologic criteria for clinical tumor response (*Therasse et al. 2000*), the response rate among patients was around 2-3% (*Rosenberg et al. 2004*).

1.6.2. Adoptive Cell Therapy (ACT)

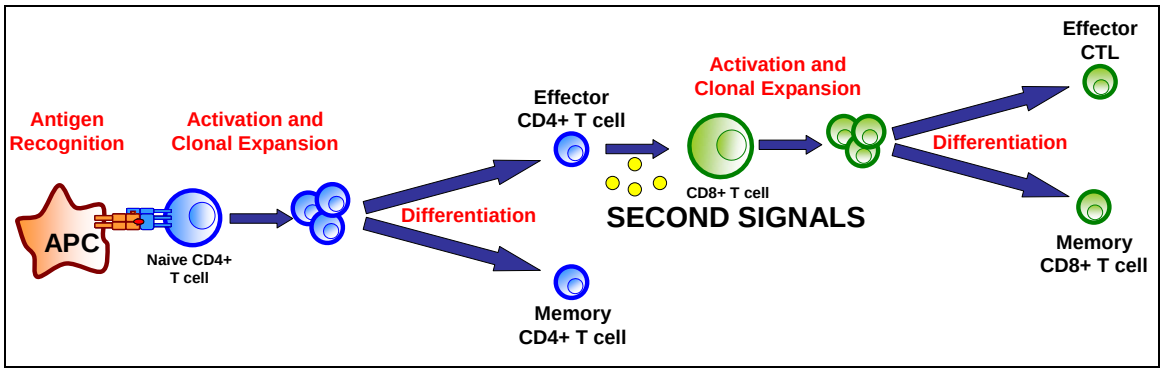
The adoptive cell therapy is a passive approach for immunotherapy and represents an important advance in cancer immunotherapy. The powerful potential of immunotherapy to mediate the regression of large volumes of metastatic disease in experimental models and in humans was confirmed in several studies. The administration of *ex vivo*–activated and –expanded autologous tumor-reactive T cells, indeed, is currently one of the few immunotherapies that can induce objective clinical responses in significant numbers of patients with metastatic solid tumors (*Rosenberg et al. 2004; Rosenberg and Dudley, 2004;* ***Errore. L'origine riferimento non è stata trovata.****and Rosenberg, 2003*). For example, ACT after

lymphodepleting conditioning caused objective responses in 46% of patients with metastatic melanoma refractory to other therapeutic modalities (*Dudley et al. 2002*), and 11% of all patients treated are complete responders (*Rosenberg and Dudley, 2004*).

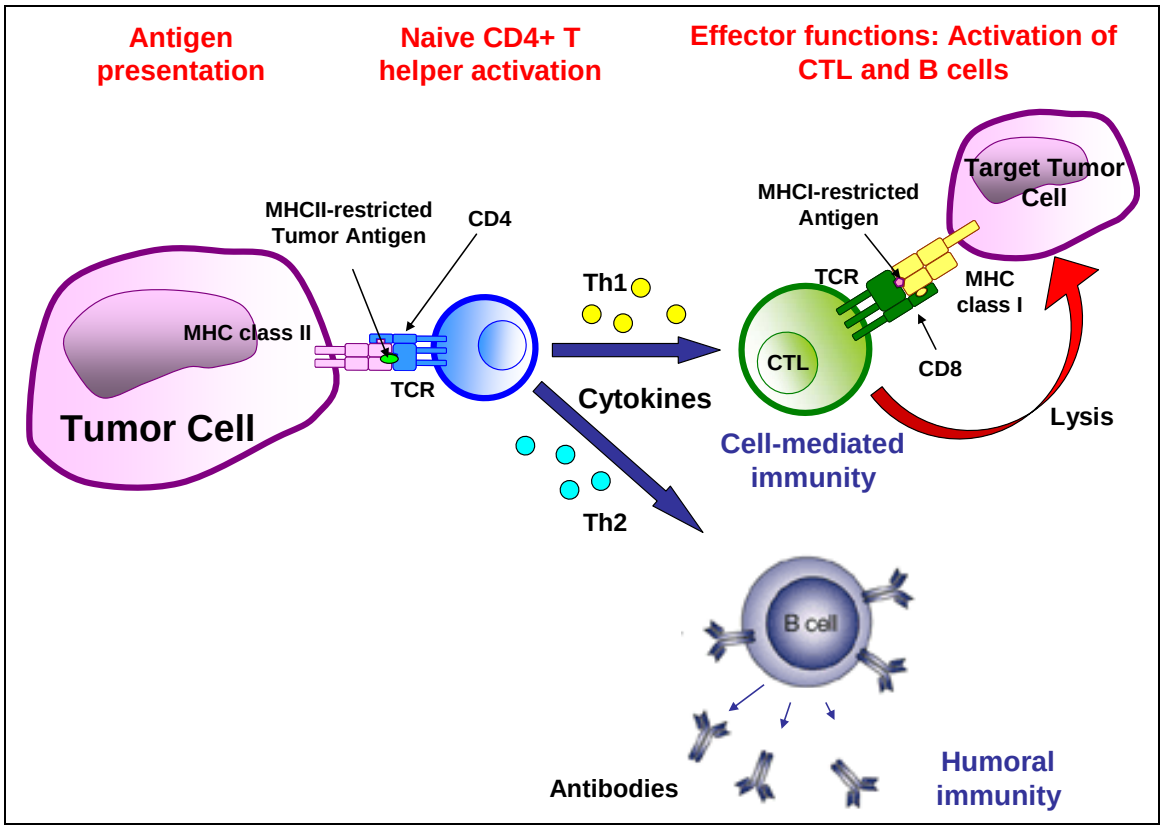
1.7 Aim of the Study

Although, as we mentioned above, it is established that the immune system can respond against tumor cells, the cancer patient clinical history often demonstrates the failure of the immune system to eliminate the tumor (*Rosenberg et al. 2004*). This is mostly due to poor tumor-specific, MHC class-II-restricted $CD4^+$ T cell (Th) help generated in tumor-bearing patients (*Hung et al. 1998; Kennedy and Celis, 2008; Muranski and Restifo, 2009*). Th cells, indeed, are required both for priming and for clonal expansion of specific CTL following re-encounter with the antigen (*Bennett et al. 1997; Schoenberger et al. 1998; Ochsenbein et al. 2001; Janssen et al. 2003; Hamilton et al. 2006*) (***Scheme 6***).

To optimize the antitumor immunological arms in terms of specificity and long-lasting memory, vaccination with tumor cells transduced with the *AIR-1*-encoded CIITA (*Accolla et al. 1986; Steimle et al. 1993*) has been explored in our laboratory. The hypothesis is that CIITA-transfected cells may act as *"surrogate APC"* for optimal triggering of tumor-specific Th cells, and thus facilitate recognition of tumor-associated antigens (TAA), presented by tumor cell MHC class II molecules. The induced MHC class II molecule expression, indeed, can elicit both the tumoricidal CTL effector mechanisms and the $CD4^+$ help mechanisms during priming, to achieve full activation and effector function of tumor-specific CTLs (*Bevan 2004*) (***Scheme 7***).



Scheme 6. Activation of effector CTLs by target-specific effector $CD4^+$ T cells, the second signal.



Scheme 7. Anti-tumoral Adaptive Immune Response.

1.7.1. Prophylactic Approach: CIITA-Cellular Vaccine

Previous results from our laboratory showed that complete rejection and long-lasting antitumor memory could be obtained after vaccination with CIITA-expressing TS/A

mammary adenocarcinoma cells both alive (*Meazza et al. 2003; Mortara et al. 2006*) and in non replicative form (*Mortara et al. 2009*). Because of the potent immunogenicity of MHC class II-positive TS/A transfectants, it became crucial to investigate whether CIITA-expressing tumor cells of different histological origin can be highly efficient immunogens as well.

The results presented here demonstrate that CIITA-expressing C51 colon adenocarcinoma, WEHI-164 fibrosarcoma, and RENCA renal adenocarcinoma, are rejected in high percentage of mice and/or strongly reduced in growth. Induction of anti-tumor immunity against poorly immunogenic tumors depended on the ability of the MHC class II-positive tumor cells to act as antigen-presenting cells (APCs) during the priming phase, processing and presenting tumor antigens to $CD4^+$ T cells, and hence to trigger $CD4^+$ T cells, which in turn induce stimulation and maturation of CTL effectors.

Importantly, the results show that immune $CD4^+$ helper T cells can induce protective antitumor responses in naïve mice injected with parental untransfected tumor cells. Since tumor rejection required also CTL, these results imply that primed $CD4^+$ T cells, with functional characteristics of T helper 1 (Th1) cells producing IFN-γ, can play a more direct role in the effector phase of tumor rejection by inducing the priming and maturation of naïve anti-tumor CTL effectors.

1.7.2. Therapeutic Approach: Adoptive Cell Immunotherapy

The other important goal in tumor immunology is immunotherapy of established tumors. Adoptive cell therapy (ACT) has been considered as a possible approach. Although at clinical level ACT results are still preliminary (*Rosenberg et al. 2008*), nevertheless the importance of

including $CD4^+$ T helper cells together with $CD8^+$ T cells to induce optimal therapeutic effects has been established (*Antony et al. 2005; Dudley et al. 2005*).

The generation of strong and reproducible anti-tumor effectors after vaccination with CIITA-tumor cells encouraged us to investigate their use in ACT immunotherapeutic approaches and to develop an efficient method to induce tumor-specific CTL *in vivo*.

In an effort to determine the components of successful immunotherapy in a relevant model of established cancer, it was decided to study the feasibility of adoptive immunotherapy approach to treat established tumor at early stages that mimic clinical situations like metastases or recidives following surgical or radiotherapy.

Antitumor efficiency of CIITA-based vaccine and adoptive therapy are dependent in part on the ability of tumor-specific T cells to persist long-term and retain antitumor function *in vivo*. Ideally, with respect to effective tumor therapy and vaccine development, such approaches would not only induce tumor regression and prevent tumor relapse, but also initiate and amplify effective endogenous recipient T cell-tumor cell interactions.

The genetic modification of tumor cells with CIITA gene could allow generation of an unlimited supply of autologous antitumor $CD4^+$ T cells that in turn could prime *in vivo* antitumor effector $CD8^+$ T cells. Our results indicate that these effectors can potently counteract tumor growth. These data will be discussed within the frame of the present knowledge of host's adaptive immune response against the tumor and in the perspective of establishing better strategies for preventing and/or treating cancer.

2. MATERIALS AND METHODS

2.1. Animal Tumor Models

BALB/c tumor cell lines, TS/Apc, mammary adenocarcinoma (kindly provided by P.L. Lollini, University of Bologna, Italy; *Nanni et al. 1983*), RENCA, renal adenocarcinoma, C51, colon adenocarcinoma (kindly provided by M. P. Colombo, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy), and WEHI-164 fibrosarcoma were cultured in DMEM supplemented with L-glutamine, HEPES, and 10% heat-inactivated fetal calf serum in a 5% CO_2 atmosphere at 37°C.

5- to 10- week-old female BALB/c (H-2K^d) mice were purchased from Harlan (Oxon, UK). Housing, treatment and sacrifice of animals followed national legislative provisions.

2.2. Plasmids and DNA transfection

The full-length human CIITA cDNA was obtained by preparative proofreading PCR (Fusion Taq; Finnzymes, Helsinki, Finland) from pREP10-CIITA plasmid, previously described (*Sartoris et al. 1996*). Forward and reverse primers bear *Xho-I* linker tails used to subclone the PCR product into the *pLXIN2.ape* retroviral vector (from Antonio Daga, Department of Translational Oncology, IST-National Cancer Research Institute, Genoa, Italy), modified from pLXIN retroviral vector (Clontech, Milan, Italy) to include additional restriction sites in the MCS.

DNA transfections were performed with *Lipofectamine™ 2000* (Invitrogen SRL, San Giuliano Milanese, Italy), according to the manufacturer's instructions. TS/A, WEHI-164,

C51 and RENCA tumor cells were transfected with 3μg of pLXIN (mock) or pLXIN-CIITA plasmid. Stable transfectants were selected in culture medium containing G418 (500μg/mL) and cloned by limiting dilution.

2.3. Flow cytometry analysis

Transfected cell lines were surface-stained with 20.8.4S anti-H2-K^d and K22–422 anti-I-E^d mAbs. A FITC-conjugated goat anti-mouse immunoglobulin (BD Becton Dickinson Italia S.p.A., Buccinasco, Milan, Italy) was used as a second step reagent. The phenotype of all cell types was analyzed by an EPICS® XL flow cytometer and EXPO32® analysis software (Beckman Coulter S.p.A., Milan, Italy).

2.4. Vaccination

Animals were injected subcutaneously with $3.5x10^6$ (WEHI-164) or $2x10^5$ (RENCA) or $1.5x10^5$ (C51) or $5x10^4$ (TS/A) parental cells or with the same dose of correspondent mock transfected cells (empty plasmid) or CIITA-expressing cells in 100μl RPMI 1640.

Tumor growth was inspected every 2-3 days, size measured by a caliper and recorded as the tumor area (mm^2).

Rejecting animals were rechallenged with the same tumorigenic dose of parental cells subcutaneously (*s.c.*) on the same flank. Experiments were performed two times with groups of four to six mice.

2.5. Adoptive Transfer (Winn Assays) for Vaccine-induced Immunity

The ability of various lymphoid spleen cell populations from CIITA-tumor vaccinated and rechallenged mice with parental tumor cells to transfer antitumor resistance to syngeneic recipients was assessed four weeks after tumor rechallenge (day 28). The spleen cells were aseptically collected from the mice, in which neither the CIITA-expressing neither the parental tumor grew, pooled for each treatment group and used for the Winn assay. $CD4^+$ or $CD8^+$ cells were purified from the total spleen cell suspension (depleted of red blood cells), by negative immunomagnetic sorting using the $CD4^+$ or the $CD8a^+$ T Cell Isolation Kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany).

The separation was performed according to the manufacturer's instruction without modification. Briefly, negative selection was performed with a pool of mAb specific for all cells except CD4- or CD8-positive cells. Purity was >95%, as assessed by immunofluorescence. The total or purified splenocytes from protected mice or age-matched parental tumor-bearing mice were admixed with viable tumor cells in a volume of 0.1 ml PBS (E:T=80:1 for C51 tumor model or E:T=1:1 for WEHI-164 tumor model). The mixture was inoculated *s.c.* at the inguinal region of BALB/c recipient mice.

2.6. Ex-vivo derived tumor cells

Suspensions of single cells, derived from outgrowing tumors in mice injected with CIITA-expressing tumor cells, were growth *in vitro* and analyzed for MHC class II expression. Briefly, biopsies of tumors were removed under sterile conditions, dissociated with a sterile syringe plunger in a cell strainer with 70μm nylon mesh (BD Becton Dickinson Italia S.p.A.,

Buccinasco, Milan, Italy), resuspended and growth in complete DMEM medium, until the time of flow cytometric analysis.

2.7. Adoptive Cell Therapy (ACT)

5–12 weeks old female BALB/c mice of age (six groups, *n* = 4–5 for group) were injected subcutaneously on the left flank with $5x10^4$ TS/A mammary adenocarcinoma cells (TS/A-pc) in 100μl of RPMI 1640. Each group was then injected, at distinct days after tumor inoculum, near the tumor inoculation sites, with $5x10^6$ immune splenocytes derived from mice vaccinated with TS/A-CIITA and rejecting TS/A parental cells.

Briefly, donor BALB/c mice were immunized against the parental tumor by injecting them *s.c.* on the left flank with $1x10^5$ CIITA-expressing TS/A cells. Boost with the same tumorigenic dose of parental TS/A (TS/A-pc) in TS/A-CIITA-rejecting animals was carried out *s.c.* on the same flank. The immunized mice were used as donors 2 weeks after the parental tumor boost. Spleens were removed under sterile conditions, dissociated with a sterile syringe plunger in a cell strainer with 40μm nylon mesh (BD Becton Dickinson Italia S.p.A., Buccinasco, Milan, Italy), treated with ACK buffer (150mM NH_4Cl; 10mM $KHCO_3$; 0.1mM EDTA; pH 7.4) and single cell suspensions resuspended in RPMI 1640. Then, tumor-bearing mice were injected with $5x10^6$ effector spleen cells suspended in 100μl of RPMI 1640.

Tumors were measured twice weekly, and sizes recorded as the tumor area (mm^2). All experiments were performed in a blinded, randomized fashion and repeated independently at least twice, with similar results. Control group for these studies included mice receiving no splenocytes. The same tumor-immune splenocytes used for adoptive immunotherapy were

admixed at 4°C in proportions of 100:1 (E:T) with TS/A-pc tumour cells and immediately thereafter, 0.2 ml of the suspension was inoculated *s.c.* on the left flank of naive animals.

2.8. Adoptive Transfer (Winn Assays) for Therapy-induced Immunity

Winn assay experiments were performed also to demonstrate antitumor protective cells in the spleen of adoptive therapy-treated tumor-bearing mice, sacrificed 30 days after tumor cells inoculum.

Naïve mice were injected *s.c.* with a mixtures of $5x10^4$ TS/A tumor cells and $1x10^7$ freshly isolated total splenocytes (E:T ratio of 200:1) from on-day-6-treated tumor-bearing mice or from control untreated tumor-bearing mice. Furthermore, to study the T cell subpopulations involved in antitumor effect, polarized $CD8^+$ and $CD4^+$ T cells were isolated from on-day-6-treated tumor-bearing mice splenocytes.

Enriched $CD8^+$ ($2x10^6$ cells) and $CD4^+$ T cells ($3x10^6$ cells) were admixed with TS/A ($5x10^4$) tumour cells in a proportion equivalent to $CD8^+$ and $CD4^+$ subtypes percentages in total spleen and inoculated *s.c.* on the left flank of naïve animals.

2.9. ELISPOT assay

Mouse IFN-γ and IL-4 ELISPOT assays were used to detect the presence of spleen cells responsive to tumor cells in BALB/c mice immunized with the CIITA-transfected cells or cured with adoptive immunotherapy. MultiScreen$^{TM}_{HST}$ 96-well Immobilon-P Membrane plates (Millipore S.p.A., Vimodrone, Milan, Italy) were coated with 100 µl of capture MM-701 Anti-Mouse Interferon-γ or MM-450D Anti-Mouse Interleukin-4 (TEMA ricerca S.r.l.,

Bologna, Italy) Purified Monoclonal Antibodies in PBS (10μg/ml) and left 2 h at 37°C. The plates were blocked with PBS-BSA 2% for 2 h at 37°C and then washed with PBS.

Responder T cells derived from the spleens of mice were added to each well ($3x10^5$ cells) in 100μl complete RPMI medium containing 10% fetal calf serum, 2mM L-glutamine, 100U/ml penicillin/streptomycin. Control wells contained responder splenocytes plus medium alone or plus Concavalin A (ConA, 10μg/ml).

Stimulator Mitomycin C-treated (60μg/ml for 2 h at 37°C) target tumor cells (10^5 cells) or gp70-derived AH1 peptide (SPSYVYHQF, synthesized by Primm S.r.l., Milan, Italy) (10μg/ml) were added to each test well at a responder:stimulator ratio of 3:1.

In certain experiments, mAb specific for either MHC class I (20.8.4S clone, 50μg/mL) or class II (K22.42.2 clone, 50μg/mL) antigens were added during the co-culture with irradiated tumor cells.

After 48h, the plates were washed with PBS-Tween20 (0.05%). Detection MM-700-B Anti-Mouse Interferon-γ or MM-450D-B Anti-Mouse Interleukin-4 Monoclonal Biotin-Labeled (2μg/ml) Antibodies (TEMA ricerca S.r.l., Bologna, Italy) were then added to each well, and the plates were left for 3 h at room temperature in the dark.

After further washing, N100 HRP-Conjugated Streptavidin (1:5,000) (TEMA ricerca S.r.l., Bologna, Italy) was added for 2h at room temperature. Colour was developed with AEC Chromogen Kit (Sigma-Aldrich Italia Srl, Milan, Italy).

The reaction was stopped after 4-6 min, with deionized water. The resulting spots were counted using a stereomicroscope.

The frequencies were then expressed as cytokine producing cells per million splenocytes. A >2-fold increase in the number of spots over the control (splenocytes cultured in the absence of tumor cells) was considered a positive response.

Data are expressed as number of spot-forming cells per million spleen cells.

2.10. CTL Activity Assay

CytoTox 96 cytotoxicity assay (Promega Italia s.r.l., Milan, Italy) was used to measure the splenocyte cytotoxic activity in treated mice according to the manufacturer's protocol with minor modification. Briefly, splenocytes were freshly isolated and incubated in the presence of Mitomycin C-treated (60μg/ml for 2 h at 37°C) tumor parental and CIITA-expressing target cells, at 50:1 effector-to-target cell ratio.

After 5 days, splenocytes were used as effector cells in the CTL assay. Parental tumor cells were used as target cells and were plated at $1x10^4$ cells per well on 96-well U-bottomed plates, and splenocytes (effectors) were added to a final volume of 100μL, in different effector-to-target cell ratios (50:1, 25:1, 12:1, and 6:1). Plates were then incubated for 4 h in a humidified 5% CO_2 chamber at 37°C and centrifuged at 500x*g* for 5 min. Aliquots (50μL) were transferred from all wells to fresh 96-well flat-bottomed plates, and an equal volume of reconstituted substrate mix was added to each well. The plates were then incubated in the dark at room temperature for 30 min. Stop solution (50μL) was then added, and absorbance values were measured at 490 nm. Cell death percentages at each effector-to-target cell ratio were calculated using [A (experimental) – A (effector spontaneous) - A (target spontaneous)] x 100 / [A (target maximum) - A (target spontaneous)].

2.11. Immunohistochemical analysis

Three mice each from TS/A tumor or on-day-6-adoptive therapy-treated TS/A tumor groups were euthanized at 14 days after *s.c.* tumor inoculation. Cryostat sections (6μm thick) were immunostained using a streptavidin-biotin-alkaline phosphatase complex staining kit (Bio-Spa Division, Milan, Italy) and naphthol-AS-MX-phosphate and Fast Red TR (Sigma, St. Louis, MO) to visualize binding sites. The mAbs used were anti-CD4 (GK1.5), anti-CD8 (2.43), anti–dendritic cells (DEC-205; clone NLDC-145; ImmunoKontact, Lugano, Switzerland), anti-polymorphonucleate Ly-6G (Gr-1; clone RB6-8C5) and anti-macrophage (clone MOMA-1; ImmunoKontact). The sections were incubated with the primary antibody overnight at 4°C. The red reaction product was obtained using a mixture of 2mg naphthol-AS-MX-phosphate dissolved in 200μL *N*,*N*-dimethylformamide (Sigma) and diluted in 9.8mL of 0.1mol/L Tris-HCl (pH 8.2) and 1 mmol/L levamisole (Sigma). Immediately before use, 10mg Fast Red TR salt was added. Gill's haematoxylin was used as a counter stain and the sections were mounted in glycerol (DAKO, Carpinteria, CA). Cell counts were obtained in 8 to 12 randomly chosen fields under a Leica Wetzlar light microscope (Solms, Germany) at x400 magnification, 0.180mm^2/field. Mann-Whitney U test was used to evaluate whether there was a statistically significant difference between TS/A and adoptive therapy-TS/A cellular infiltrate. Data analyses were considered highly significant when $P < 0.005$.

2.12. Statistical Analysis

Mean values ±SEM were calculated for each group at each time. Average tumor sizes were plotted over time to display the trend of tumor development in each treatment group. All statistical analyses were done using the Student's *t* test to evaluate significance between

groups, except that the log-rank statistics was used to test differences between groups. For all cases, results were regarded significant if P values were <0.01 or <0.05. All *in vivo* experiments were performed two times with groups of four to six mice, and the results were cumulated as they gave homogeneous results.

3. RESULTS

3.1. Rejection of CIITA-tumors and protective immunity against parental tumors.

To evaluate the ability of naïve mice to reject CIITA-expressing tumors, tumor cell lines of different histotype that causes 100% mortality in BALB/c mice were used. As shown in ***Figure 1A***, in these tumor cell lines, the absence of MHC class II gene transcription correlates with the lack of CIITA expression. The transfection of CIITA gene, indeed, induces *de novo* expression of MHC class II molecules into the tumor cell lines. Morphology and growth kinetics *in vitro* of transfected cells did not differ with respect to untransfected parental cells (data not shown).

When CIITA-transfected tumor cells were injected *in vivo*, complete rejection or significant delay in tumor growth was observed in all tumor models, when compared to parental tumors and to tumor cells transfected with the mock vector (***Figure 2A-B***, *$P<0.01$). CIITA expression indeed can inhibit tumor growth in 100% of animals implanted with WEHI-CIITA cells and 80% of animals implanted with C51-CIITA cells, contrary to parental WEHI and C51 cells for which tumor take was 100%. Importantly, in the remaining 20% of C51-CIITA injected mice, tumors developed much later as compared to C51 parental tumors. Although not completely rejected, the RENCA-CIITA tumor grew more slowly than did tumors induced by the parental cells, suggesting that the immune response generated was ineffective for a complete tumor rejection (***Figure 2A-B***).

Capacity to reject the tumor correlated with the amount and stability of CIITA and thus corresponding MHC class II expression (*Meazza et al. 2003*). Indeed, *ex vivo*-isolated cells from C51-CIITA tumor-bearing mice showed decreased MHC class II expression; more importantly *ex vivo*-isolated cells from RENCA-CIITA tumor-bearing mice, which displayed only retarded tumor growth, showed an almost complete loss of MHC class II expression (***Figure 1B***). The reasons of the reduced stability of CIITA expression *in vivo* in RENCA cells are under investigation.

It was next evaluated whether initial vaccination with the CIITA-expressing tumor cells would induce a memory response against the parental cells. Thirty days after vaccination, tumor-free mice were challenged with parental cells. 100% of animals vaccinated with WEHI-CIITA and with C51-CIITA showed complete rejection. This protective response was long lasting, since 90 days post-vaccination animals were still able to reject a second tumor challenge (***Table 2***). Thus, vaccination with CIITA-mediated MHC class II expressing tumors results in long lasting, specific anti-tumor immunity against MHC class II negative parental tumor cells.

3.2. Role of $CD8^+$ effector and $CD4^+$ T helper cells in CIITA-Vaccine approach.

To verify whether the effector function in challenge experiments of tumor rejection was mediated by specific lymphocyte subpopulations, $CD4^+$ and $CD8^+$ spleen cells of mice rejecting the CIITA-tumor vaccine and a parental tumor challenge were used to protect naïve animals in adoptive transfer experiments. These experiments were performed using total spleen cells or the equivalent proportion of purified $CD4^+$ or $CD8^+$ tumor-immune

splenocytes. Mice were inoculated with effector (E) and tumor (T) cells, with an 80:1 (E:T) proportion for C51 cells and a 1:1 (E:T) proportion for WEHI-164 cells.

As shown in ***Figure 3***, unselected spleen cells for both WEHI-CIITA and C51-CIITA vaccinated and challenged mice protected 100% of injected mice from tumor growth. Interestingly, purified $CD4^+$ T cells were extremely potent in protecting mice from WEHI tumor take, as 80% of the animals were protected and the remaining 20% manifested a retarded growth (***Figure 3A,*** upper panels). Purified $CD4^+$ T cells were also efficacious in preventing tumor growth of C51 tumor, as 50% of the animals were protected and the remaining 50% displayed a significant tumor growth retardation (***Figure 3A,*** lower panels). Similar results were obtained when immune $CD8^+$ T cells were used in adoptive transfer; in this case 60% of WEHI injected mice (***Figure 3A,*** upper panels) and 100% of C51 injected mice were protected (***Figure 3A,*** lower panels).

These results strongly indicate that both $CD4^+$ and $CD8^+$ T cells can act as effectors against the tumors if previously triggered by MHC class II-positive tumor cells. It was subsequently assessed whether mice resistant to tumor take could reject a tumor challenge after 1 month from adoptive cell transfer. Animals rejecting WEHI tumors after total spleen cell, $CD4^+$ or $CD8^+$ T cell transfer were able to reject in 100% of the cases a tumor rechallenge (***Figure 3B,*** left panel). Similarly, mice rejecting C51 tumors after total spleen cells or $CD4^+$ T cell transfer acquired immunological memory and rejected in 100% of the cases a tumor rechallenge, whereas only 50% of mice rejecting the tumor after $CD8^+$ cell transfer were resistant to a further rechallenge (***Figure 3B,*** right panel)

These results demonstrate that CIITA-induced tumor-immune T cells protect naïve mice and that the antitumor responses induced with CIITA-based vaccination is dependent mostly on $CD4^+$ and $CD8^+$ T cells, able to induce also alone transplantable memory responses. Of great

importance, it was shown also that not only CIITA-vaccine-induced $CD8^+$ CTLs, but also $CD4^+$ helper T cells can induce effector antitumor responses. Finally, it was also shown that this anti-tumor memory is long lasting, as 90 days after the first challenge tumor-free animals in the all three groups are still able to reject a second challenge with parental cells (data not shown).

3.3. CIITA-tumor-mediated $CD4^+$ T cell priming and generation of anti-tumor CTLs

$CD8^+$ CTL are major effectors in killing the tumor cells *in vivo*. However, as show above in our experimental system primed $CD4^+$ T cells transferred into naïve recipients can protect the animals likewise. To assess the importance of $CD4^+$ T cells in the generation of anti-tumor $CD8^+$ cytolytic activity, we investigated the CTL responses in both WEHI-CIITA and C51-CIITA vaccinated mice, 4 weeks after vaccination, and in naïve mice receiving adoptive cell transfer of anti-tumor immune lymphocyte subpopulations.

Mice were vaccinated with CIITA-expressing WEHI or C51 tumor cells, as described above. At week 4, splenocytes from the mice in each group were harvested and pooled. CTL responses specific to tumor cells were measured following antigen restimulation *in vitro*. Vaccinated mice developed a strong CTL activity (***Figure 4A,C***). A second interesting step was determining if vaccination can induce CTL responses able to be transplanted in naïve mice. As shown in ***Figure 4B*** and ***Figure 4D***, after adoptive transfer, CTL activity was higher in animals that received $CD4^+$ T cells as compared to animals receiving $CD8^+$ T cells. Indeed, CTL activity generated by $CD4^+$ T cell transfer was as high as the one generated by total spleen cell transfer for both WEHI and C51 tumors. CTL activity was strongly reduced in

presence of anti-MHC class I, but not anti MHC class II, antibodies (data not shown). These results strongly suggest that anti-tumor primed $CD4^+$ T cells generated by the vaccination procedure with CIITA-transfected tumors support the activation of naïve $CD8^+$ T cells to become fully differentiated anti-tumor CTLs.

3.4. ACT by CIITA-cell vaccine induced immune effectors

We subsequently investigated whether anti-tumor immune lymphocytes could be used for adoptive cell therapy (ACT) of established tumors. For this approach we used effectors generated in the more extensively studied TS/A-CIITA vaccination system (*Meazza et al. 2003; Mortara et al. 2006; Mortara et al. 2009*). Vaccination experiments were repeated with TS/A-LXIN-CIITA tumor cells (data not shown) and confirmed the efficacy of TS/A-LXIN-CIITA clone to induced effective protection *in vivo*, fully comparable to our previous results with TS/A-CIITA 32.10.7 clone (*Mortara et al. 2006*). Donor TS/A-LXIN-CIITA-rejecting mice were boosted one time with TS/A-pc tumor cells, 2-weeks before the adoptive transfer in tumor-bearing mice.

Treatment with tumor-immune splenocytes, up to day 6 after tumor inoculum, was efficient in curing or significantly retarding tumor growth. As showed in ***Figure 5A,B***, all untreated mice developed tumors, but, at day 0, day 1 and day 3 after tumor inoculum, immune spleen cells (ACT day 0, 1, and day 3) completely and stably cured 100%, 75% and 50% of the tumors, respectively, and strongly retarded tumor growth in the remaining 25% (day 1) and 50% (day 3) of tumors. Injection of immune cells at day 6 (ACT day 6), time in which mice displayed already an evident tumor mass, although not curing, was instrumental in retarding tumor growth as observed in the non cured mice after ACT at day 1 and 3. Injection of immune

spleen cells 12 days after tumor inoculum (ACT day 12) was instead inefficient either in protecting or retarding the growth of the subcutaneously inoculated mammary adenocarcinoma. Animals injected with splenocytes did not show signs of discomfort and their mean body weight did not differ from that of the controls (data not shown).

Cures were defined as those animals that were tumor-free after treatment for at least 30 days. It was then investigated whether tumor rejection generated by ACT (i.e., day 0, day 1 and day 3) was long-lasting. Cured animals were rechallenged with TS/Apc 30 days after ACT to determine the presence of immunologic memory.

All ACT-protected mice rejected the subsequent parental tumor challenge (***Table 3***), indicating again the capacity of transferred immune cells to persist *in vivo* and retain immunological memory.

3.5. Therapeutic effect of T-cell subsets.

The fact that a strong retardation of tumor growth was achieved by ACT after 6 days from tumor inoculum prompted us to assess whether immune effectors from these animals were able to influence the tumor take and/or growth when coinjected with tumor cells into naïve animals.

Total spleen cells (Tot sc), were fully competent in protecting naïve mice from tumor take in 100% of the animals (***Figure 5C,D***). Interestingly, $CD4^+$ T cells fully protected 60% of the animals and strongly retarded tumor growth in the remaining 40% of mice.

On the other hand, $CD8^+$ T cells were incapable of fully protecting the animals, although they were still able to strongly delay tumor growth. As negative control, the adoptive transfer of

total splenocytes from not treated TS/A tumor-bearing mice (C sc) did not affect tumor growth. Thus, immune lymphocytes, and particularly $CD4^+$ T cells had a stronger anti-tumor protecting activity than $CD8^+$ lymphocyte effectors.

3.6. CTL responses in ACT-treated mice.

TS/A tumor-bearing mice were treated with CIITA vaccine-induced tumor immune spleen cells as described above. After 4 weeks, splenocytes from mice of each group (0, 1, 3, 6, 12 days) were taken and CTL responses specific to tumor cells were measured, following antigen re-stimulation *in vitro*.

Representative results, referred to spleen cells of ACT at day 3 and day 6, are shown in ***Figure 6A***. The group of cured mice (ACT day 3) developed a higher level of parental tumor-specific lytic activity than those injected with parental tumor cells alone (Untreated tumors). Those mice that were not cured, but had significantly reduced tumor growth kinetics, displayed a reduced but still significant cytolytic activity (ACT at day 6).

We then assessed the CTL response of spleen cells of mice that were protected when coinjected with tumor cells and total spleen cells from ACT day 6-treated animals (refer to ***Figure 5C*** and ***Figure 5D***).

Interestingly, a very strong CTL activity was observed in these mice (***Figure 6B***), perhaps because these CTL responses were less anergized by tumor microenvironment and gave very high cytotoxic signals compared to ACT at day 6 in ***Figure 6A***.

Taken together, these results show that the anti-tumor effector response, generated in mice undergoing adoptive cell immunotherapy, was only quantitatively, but not qualitatively, insufficient in controlling and counteracting tumor growth.

3.7. Adoptive cell therapy induces a massive infiltration of TS/A tumors by CD4$^+$ T cells, CD8$^+$ T cells, dendritic cells and granulocytes.

In order to assess the modifications induced by ACT in the tumor microenvironment we then studied by immunohistochemistry the tumor tissue and particularly the phenotype and number of infiltrating leukocyte subpopulations in mice undergoing ACT at day 6, and specifically 8 days after ACT (14 days after tumor inoculum). At this time point massive infiltration of CD4$^+$ T cells, CD8$^+$ T cells and dendritic cells was observed in TS/A tumor masses as compared with the virtually absent representation of these lymphocyte subpopulations in the tumor of mice not undergoing ACT (***Figure 7A***). On the other hand, macrophages, which were observed at discrete number already in the untreated parental TS/A tumor microenvironment, were not statistically increased after ACT. Importantly, instead, a very significant increase of granulocytes (PMN) was present in the tumor tissue after ACT. Although this experiment did not allow to establish exactly the origin of the leukocyte infiltrate, i.e. whether exclusively deriving from ACT cells or also from host cells, it is certain that PMN infiltration is of exclusive host derivation, as only splenic lymphocytes and monocytes from immune mice were used in the ACT regimen. However it appears plausible that most of the lymphocyte subpopulations, particularly the CD8$^+$ CTL effectors were from the ACT derivation, since already at this very early time point the tumor microenvironment

displayed extensive areas of necrosis, as shown in ***Figure 7B*** (necrotic area -nec- is depicted). A very similar aspect of the tumor tissue was observed in mice undergoing ACT at day 0, 1 and 3, with even more dramatic increase in the number of necrotic areas observed (data not shown), compatible with the superior rejecting capacity of ACT at these time points.

3.8. Tumor-specific mixed Th1-Th2 immune responses after ACT.

To get further information on the characteristics of immune responses taking place in ACT-treated mice, we investigated tumor-specific IFN-γ- and IL-4-producing spleen cells, at 4 weeks from tumor inoculum, in tumor-rejecting mice after day 3 ACT, or of mice with delayed tumor growth after day 6 ACT (***Figure 8***).

Increased frequency of tumor-specific, IFN-γ-producing spleen cells was observed in tumor-cured mice (***Figure 8A***, left panel, ACT tum$^-$) but not in day-6 ACT treated mice (***Figure 8A***, left panel, ACT tum$^+$) when splenocytes were re-stimulated *in vitro* with TS/A-CIITA cells, TS/Apc or the AH1 peptide.

The number of spleen cells producing IFN-γ in response to *in vitro* stimulation with AH1 peptide, an immune-dominant peptide of the gp70 env glycoprotein of an endogenous retrovirus recognized by MHC-I-restricted CTLs on TS/A tumors (*Huang et al. 1996*), was higher in tumor-free than in tumor-bearing mice (***Figure 8A***).

Interestingly, the IFN-γ-specific response of tumor-cured mice was strongly inhibited by anti-MHC-II antibodies and, to much lesser extent by anti-MHC-I antibodies, suggesting a Th1-type response attributable mostly to CD4$^+$ T cells (***Figure 8A***, right panel, ACT tum$^-$).

The frequency of IL-4-producing cells was also increased in tumor-cured mice particularly when spleen cells were restimulated *in vitro* with TS/A-CIITA and parental TS/A tumor, but not with the AH1 peptide (***Figure 8B,*** left panel, ACT tum$^-$).

Moreover and at variance with results found for IFN-γ producing cells, significantly higher numbers of IL-4 producing cells were also observed in ACT tumor-bearing mice (***Figure 8B***, left panel, ACT tum$^+$) when restimulated *in vitro* with all three stimuli used. Also in this case the major IL-4-producing spleen cells in both tumor-cured and slow-growing tumor-bearing mice was predominantly attributable to CD4$^+$ T cells, as it was strongly inhibited by anti-MHC-II, and not anti-MHC-I, antibodies (***Figure 8B***, right panel, ACT tum$^+$).

These results indicate that a mixed Th1-Th2 type of response, mainly attributable to CD4$^+$ T cells, was present in animals cured by ACT.

On the other hand, animals, which could not be cured by ACT, although displaying slow-growing tumors, had a predominant CD4$^+$ Th2-type of response. These antitumor immune responses *in vivo* are very persistent, since we can detect it in the spleen cells of cured mice also after 1 month from tumor injection.

3.9. Tumor-specific mixed Th1-Th2 immune responses after Winn Assays

It was therefore important to assess whether the polarization of the immune response in day 6 ACT-treated mice was conserved when spleen cells of these mice were used in adoptive cell transfer admixed with tumor cells, as we knew these cells were capable to fully protect from tumor take (*see* ***Figure 5B***). Interestingly, high frequency of both IFN-γ- (***Figure 9A***) and IL-4-producing (***Figure 9B***) cells were detected in the spleen of these animals when stimulated

in vitro by all stimuli including TS/A parental tumors, the AH1 MHC class I-restricted peptide and, particularly, TS/A-CIITA cells. In both cases, responses were strongly inhibited by incubation with anti-MHC-II antibodies and to lesser extent, with anti-MHC-I antibodies (***Figure 9A-B***, right panels). These results indicate a re-polarization toward a mixed Th1/Th2 phenotype of the tumor-specific T cells that, at the origin, were mostly polarized toward a Th2 phenotype (see ***Figure 8A-B***, ACT tum^+).

4. DISCUSSION

The aim of this investigation was two fold. First, to assess whether efficacious anti-tumor response could be generated against highly tumorigenic cells of different histological origin by the CIITA transfection approach, as this is important for envisaging application of this vaccination strategy in human settings. Second, to explore the possibility of using immune, tumor-specific lymphocytes generated by the CIITA-tumor vaccination approach as immunotherapeutic tool for established cancers.

As far as the first aim, the results presented here unambiguously show that, in addition to the already described TS/A mammary adenocarcinoma (*Meazza et al. 2003; Mortara et al. 2006; Mortara et al. 2009*), tumors as different as colon carcinomas (C51) and sarcomas (WEHI-164) could be rendered highly immunogenic and rejectable *in vivo* by CIITA-mediated gene transfer. This marked therapeutic effect, confirmed in three different tumor models, not only produces a reduction in tumor size, but results in a significant number of cured animals.

The results provide unprecedented preclinical experimental data indicating that poorly immunogenic tumor cells of different histotype are rejected if they express MHC class II antigens induced by stable transfection of the MHC class II transactivator CIITA.

Interestingly, although CIITA-RENCA tumor cells could not be fully rejected, they grew *in vivo* with a significantly reduced kinetics as compared to parental untransfected cells. This correlated with the reduction of CIITA-mediated MHC-II expression of the tumor arising *in vivo*, as we have shown previously also in the TS/A tumor model (*Meazza et al. 2003*). Thus, the presence of CIITA-induced MHC-II molecules in tumor cells is instrumental to trigger antigen-specific antitumor response capable of protecting *in vivo* the vaccinated animals from

tumor take, and, more importantly, to protect them against further rechallenge with parental MHC-II negative tumor cells. CIITA-tumor vaccinated mice, indeed, potently reject parental untransfected tumors and generate tumor-specific, long –lasting T cells of superior action in adaptive immunotherapy.

CIITA-induced MHC-II-positive tumor cells may act themselves as surrogate antigen presenting cells since they have this capability (*Mortara et al. 2006*), or they may be engulphed as necrotic and/or apoptotic bodies by dendritic cells and *"fuel"* dendritic cells with preformed MHC-II-antigenic peptide complexes with additional and superior stimulating capacity for $CD4^+$ tumor-specific T helper cells, as it has been recently suggested (*Mortara et al. 2009; Dolan et al. 2006*). Whatever the mechanism, it appears that CIITA, via its induction of tumor cell MHC-II expression, may act as a specific *"adjuvant"* (*Kim et al. 2008*), inducing an amplification of the response induced by the TAA, thus bypassing the need of identifying the TAA relevant for the tumor rejection.

The importance of $CD4^+$ T cell priming in generating and maintaining efficient anti-tumor response and tumor rejection after CIITA-tumor vaccination, was witnessed by the fact that immune $CD4^+$ T cells alone could protect naïve animals from tumor take in adoptive cell transfer assays both in the case of WEHI-164 and C51 tumors. This protection was accompanied by the elicitation of strong cytolytic activity mediated by $CD8^+$ CTL and was observed not only in spleen cells of CIITA-tumor vaccinated mice but also in spleen cells of naïve mice adoptively transferred with immune $CD4^+$ T cells and challenged with parental tumors (***Figure 3***). Indeed, in these latter mice CTL activity was even stronger than the one in similar mice which received immune $CD8^+$ T cells. These results reiterate the importance of anti-tumor specific, primed $CD4^+$ T cells in triggering naïve anti-tumor $CD8^+$ T cell precursors to become fully differentiated functional CTLs as we have recently observed also

in a different model of therapy-induced tumor vaccination (*Balza et al. 2006; Mortara et al. 2007*). Importantly, these results also demonstrate that, once primed, $CD4^+$ T helper cells do not need MHC class II expression on tumor cells to be restimulated, strongly suggesting that their restimulation and survival *in vivo* can be maintained by a minimal requirement of tumor antigen availability, possibly fulfilled by MHC-II-restricted antigen presentation accomplished by classical APCs having engulphed tumor cells as necrotic and/or apoptotic bodies, as suggested above.

The other important aim of this project was to assess whether tumor-specific lymphocytes generated by CIITA-tumor vaccination could be used as immunotherapeutic tool for established tumors.

Although preliminary success in several experimental models served as background to apply the adoptive cell therapy (ACT) to human settings (reviewed in *Rosenberg et al. 2008*), results in terms of cure and/or diminution of tumor masses were unsatisfactory, with a possible exception of metastatic human melanomas (*Dudley et al. 2008*). Nevertheless, currently ACT approach seems to offer better applicability with respect to strategies such as non-specific immune modulation, active immunization with TAA or with a wide variety of immunizing vectors although the identification of the best *"effector"* for ACT and thus, in turn, the best immune stimulation of it still remains an unresolved issue. Within this frame, it seems that the polyclonal nature of anti-tumor immune response and the presence of $CD4^+$ T cells are fundamental to mediate tumor rejection (*Pardoll et al. 1998; Cerundolo , 1999; Pardoll et al. 2001; Yee et al. 2002; Dudley et al. 2005; Moeller et al. 2005; Perez-Diez et al. 2007*).

In previous studies, we have found that both $CD4^+$ and $CD8^+$ T cells collaboratively participated in mediating the regression of the murine CIITA-expressing TS/A mammary adenocarcinoma (*Mortara et al. 2006*). The results presented here indicate that immune

effectors, $CD4^+$ T helper cells and $CD8^+$ CTLs, generated by vaccination with CIITA-transfected tumors are capable to cure and/or significantly reduce the growth of established tumors in the highly aggressive TS/A tumor model.

Of great importance, the T cells obtained from vaccinated mice were therapeutic also without other *in vitro* activation passages, like treatment with anti-CD3 and IL-2, differently to some other approaches of vaccination and ACT (*Winter et al. 2003; Tanaka et al. 1999*). The first finding of note, indeed, is that ACT with whole immune spleen cells undergoing neither treatment nor amplification *in vitro* can still be totally curative in 50% of the animals at day 3 after tumor inoculum and highly effective in reducing the growth at day 6 after tumor inoculum. The negative regulation of the anti-tumor immune response in the less responsive animals may depend on the size of the tumor. Spleen cells from BALB/c mice that were treated at day 6, indeed, were shown to be still capable of induce the complete protection against TS/A tumor cells in Winn Assay experiments (***Figure 5C,D***).

Furthermore, ACT induced proliferation of tumor-specific immune splenocytes in receiving mice, leading to rejection of subsequent tumor challenge or to protection from metastasis in animals having established tumors. The second finding of note, indeed, is that anti-tumor lymphocytes were long-living cells since cells even from day 6 ACT animals, taken one month after therapy, were extremely efficient in protecting naïve animals from tumor take in adoptive cell transfer assays. Of great importance, this transfer of the tumor-specific immunity did not require any other manipulation of the recipient animals, contrary to other studies in which hosts received adoptively transferred cells after host lymphodepletion treatment. Depletion of immune cells before adoptive cell transfer, indeed, can markedly improve the antitumor efficacy of transferred cells, due to several different mechanisms (*Gattinoni et al. 2006*), as the creation of niches for the transferred cells and the elimination of

regulatory T cells and endogenous cells that limit homeostatic proliferation of the transferred cells, due to competition for activating cytokines, known as the *"cytokine sink"* effect (*Gattinoni et al. 2005; Klebanoff et al. 2005*).

The third finding of note is that $CD4^+$ T cells were remarkably more efficient that $CD8^+$ T cells in conferring protection from tumor take.

The latter finding, along with the fact that a potent anti-tumor CTL response was present in these animals (***Figure 6***), further re-emphasizes the key role of tumor-specific helper signals from CIITA-vaccine primed $CD4^+$ T cells in triggering and maintaining the effector function of $CD8^+$ CTL naïve precursors, in tumor-bearing ACT treated mice although we cannot exclude that primed $CD4^+$ T cells may include as well a subset of cytotoxic $CD4^+$ T cells (*Cohen et al. 2000*). Future studies will further clarify this aspect.

Assuming that Th1 and Th2 $CD4^+$ T cells have been shown to have distinct roles in various immune responses (*Nishimura et al. 1999*), including antitumor immune responses, further phenoytypic and functional characterization of the lymphocyte subpopulations present in mice undergoing ACT was performed. It was shown that spleen cells from tumor-cured mice displayed a mixed Th1/Th2 phenotype with increased frequency of both IFN-γ ($CD4^+$ and $CD8^+$ T cells) and IL-4 ($CD4^+$ T cells) producing cells, whereas spleen cells from day6 ACT mice with retarded tumor growth exhibited a prevalent Th2 phenotype. Interestingly, this latter Th2 phenotype switched to a mixed Th1/Th2 phenotype with a strong IFN-γ and IL-4 type of response when these cells were used for adoptive transfer in virgin animals, in which they caused protection from tumor take. The only apparent distinction with the mixed Th1/Th2 phenotype observed in ACT tumor-cured mice was the maintenance of the AH1 peptide-specific component of response in the Th2 type cells. The reasons of this differential response are presently matter of investigation. It is important to stress however the fact that

switch from a prevalent Th2 to a mixed Th1/Th2 type of response was accompanied by a strong increase of cytolytic activity in these spleen cells.

Taken together, these data strongly suggest that the major anti-tumor immune cells generated by our vaccination approach and used for ACT therapy display a modulatable phenotype and, depending upon their interaction with the tumor microenvironment and their relative proportion with respect to tumor cells, they can re-polarize assuming a more protective phenotype when they are Th1 (*Mortara et al. 2006*) or both Th1/Th2 (*Mortara et al. 2009*) with respect to a less protective phenotype when they are, or they are forced to be, only of the Th2 phenotype. Several examples of a mixed Th1/Th2 immune response correlating with the tumor rejection have been reported (*Hung et al. 1998; Schuler et al. 1999; Lopez et al. 2005*).

Interestingly, we recently found that a protective Th1/Th2 type of immune response was also observed in mice protected from tumor growth after a therapeutic procedure that targets tumor vessels and result in intense necrosis of tumor tissue (*Balza et al. 2006; Mortara et al. 2007*). Thus, it is likely that plasticity of immune effectors, particularly $CD4^+$ T helper cells, is at the basis of a protective anti-tumor immune response and provides these cells were originally triggered by optimal antigen presentation generated, as in our approach, by CIITA-dependent MHC class II expression in tumor cells.

The immunotherapeutic strategy described here uses autologous tumor cells as the immunogen. Not all human malignancies will be amenable to this approach, since not all cancers can be cultured or transduced *in vitro*. However, phase I/II clinical trials using autologous tumor cells as immunotherapeutic agents are currently underway for a variety of human tumors [neuroblastoma, breast, melanoma, prostate, glioma, papilloma (http://cancernet.nci.nih.gov)].

Some results strongly suggest that combination therapies, such as those involving vaccination combined with adoptive T-cell transfer, may produce synergistic antitumor responses that are considerably more potent than either approach used in isolation (*Overwijk et al. 2003; Parviz et al. 2003; Lou et al. 2004; Jorritsma et al. 2008; Tamai et al. 2008; Chen et al. 2009*).

Within this frame, vaccination and therapeutic approaches, both directed to induce specific anti-tumor immune responses, are proposed. These approaches are based on CIITA-genetically engineering tumor cells that become more *"immunogenic"* and thereby stimulate *in vivo* the generation of tumor-specific effector cells. Results from these strategies suggest that the appropriate polarization of effector $CD4^+$ T cells induced by vaccination, could enhance the successful tumor eradication by adoptive immunotherapy, after appropriate amplification strategies *in vitro* of tumor-specific vaccine-induced $CD4^+$ T cells (*Caserta et al. 2009*).

In conclusion, tumor cell modification by CIITA may offer an alternative strategy not only for preventive vaccination in a wide variety of tumors but also for the generation of superior anti-tumor immune lymphocytes for adoptive cell therapy.

5. FIGURES and TABLES

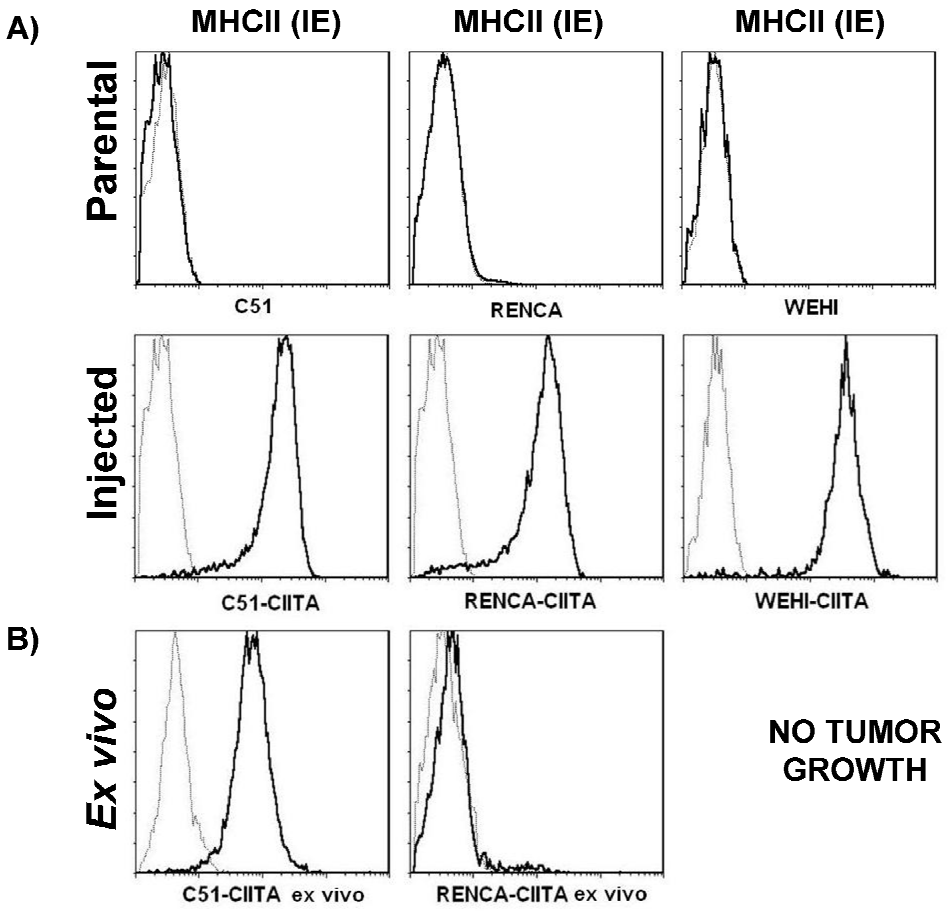


Figure 1. CIITA-dependent MHC-II expression on tumor cells of different histotype.

A). Immunofluorescence flow cytometry profiles of CIITA-transfected C51 colon adenocarcinoma, RENCA renal carcinoma and WEHI-164 fibrosarcoma tumor cells stained for MHC class II molecules (darker histograms) as compared with untransfected controls (lighter histograms). *B).* MHC class II phenotype of *ex-vivo* tumor cells from C51-CIITA and RENCA-CIITA showing that these tumor cells have a reduced expression of MHC class II cell surface molecules as result of *in vivo* down regulation of expression transfected CIITA. Cells were stained by indirect immunofluorescence with mAb specific for I-E (K22.422) *plus* FITC-conjugated goat anti-mouse mAb. Dashed histograms represent negative control of an irrelevant isotype-matched mAb.

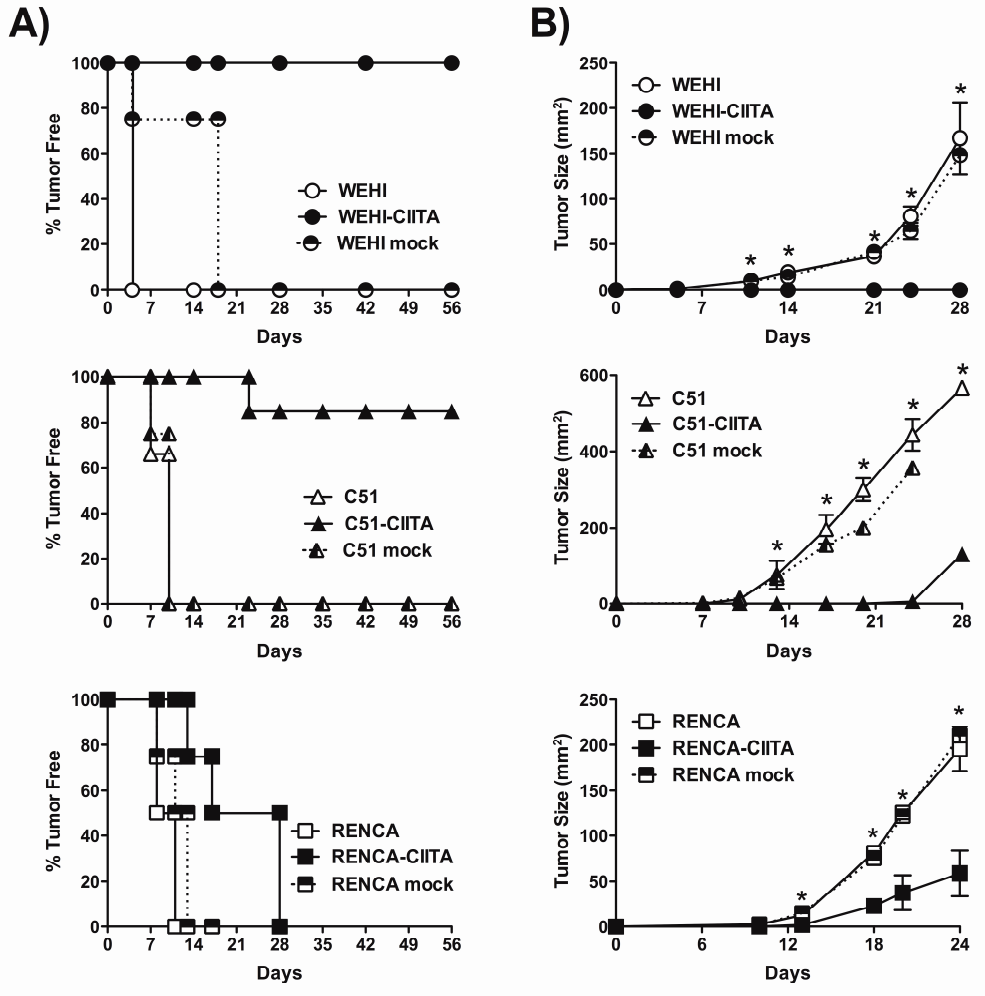


Figure 2. CIITA-tumor cell lines are rejected or drastically retarded in their growth in vivo.

Equal numbers of transfected or parental tumor cells were injected subcutaneously (*s.c*) in BALB/c mice. At least 8 animals were injected per group and one representative experiment of at least two is shown for each tumor model. *A)* Tumor growth was monitored over time (abscissa) and results expressed as percentage of tumor-free mice in the ordinate. *B)* Tumors arising from CIITA-transfected cells, mock-transfected cells or from parental cells were compared for their corresponding size over time (abscissa) and results expressed as tumor size (mm^2) in the ordinate. Data represent means ± SEM of tumor volumes and significant differences from parental tumor group are indicated (*P<0.01). Open symbols refer to parental untransfected tumor cells; full symbols refer to CIITA-transfected tumor cells.

Table 2. Mice rejecting CIITA-tumors are resistant to challenge with parental tumor and develop long-lasting immunological memory.

1st injection	**no. mice injected**	**% rejecting mice**	**1st challenge† (30d)**	**% rejecting mice**	**2st challenge† (90d)**	**% rejecting mice**
WEHI-CIITA	10	100 (10/10)*	WEHI-164	100 (10/10)	WEHI-164	100 (10/10)
C51-CIITA	10	80 (8/10)	C51	100 (8/8)‡	C51	100 (8/8)‡

* in parenthesis, number of animals injected with CIITA-transfected tumor / number of rejecting mice.

† first and second challenge with corresponding parental tumors were performed after 30 days and 90 days, respectively, from injection of CIITA transfected tumors.

‡ 8 out of 10 mice rejected C51-CIITA tumors. All these mice were resistant to a first and a second challenge with parental C51 tumor cells.

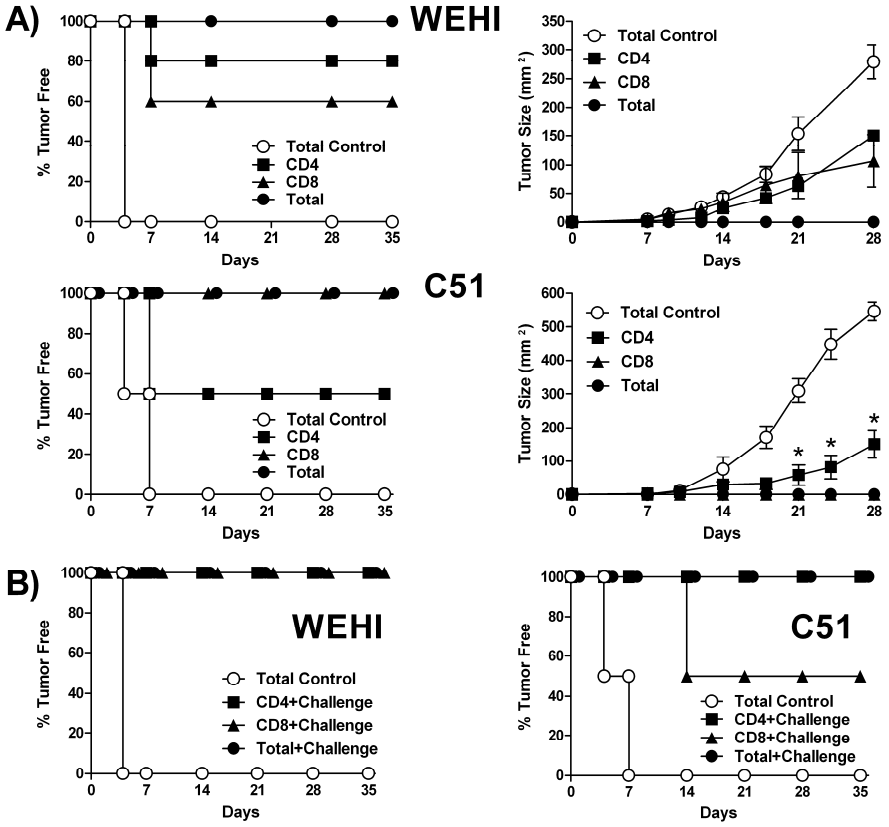


Figure 3. Role of T lymphocyte subpopulations in anti-tumor immunity after CIITA-vaccine.

A) Naïve mice were co-injected *s.c.* with immune total spleen cells, $CD4^+$ or $CD8^+$ T cells and parental tumor cells at ratio of 80:1, 40:1, 20:1 (C51) or 1:1, 0,8:1, 0,4:1 (WEHI-164), respectively. Control mice (Total control) were co-injected with total splenocytes from naïve animals and tumor cells at ratio 80:1 (C51) and 1:1 (WEHI-164). *B)* Tumor-rejecting mice after injection of listed immune cells (see A) were challenged after 30 days with parental C51 or WEHI-164 tumor cells. Total control, naïve animals challenged with tumor cells only. Tumor growth was monitored over time (abscissa) and the results expressed as percentage of tumor-free mice in the ordinate (A and B, left panels, B right panel). Tumors arising in adoptively transferred animals were compared for their corresponding size over time (abscissa) and the results expressed as tumor size (mm^2) in the ordinate (A, right panels). Data represent means ± SEM of tumor volumes and significant differences from parental tumor group are indicated (*P<0.01). At least 8 animals were included per group and one representative experiment of at least two is shown for each tumor model.

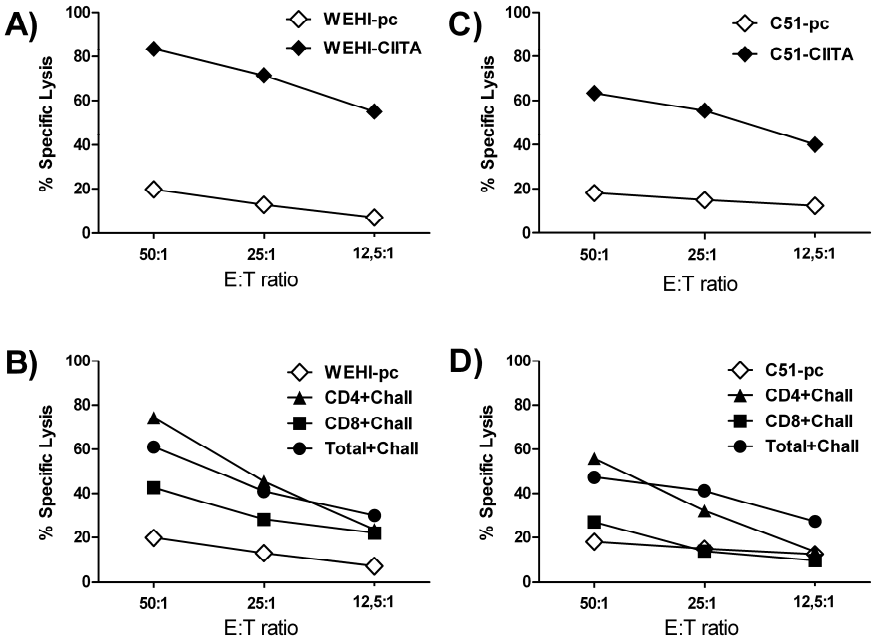


Figure 4. CIITA-vaccine generates potent CTL responses.

Splenocytes from mice rejecting CIITA-transfected tumors and further resistant to challenge with parental tumor (WEHI-CIITA or C51-CIITA), or from mice with growing tumors (WEHI pc or C51 pc) were taken 20 days from challenge, or at 20 days after tumor inoculum, respectively (A and B). Similarly, spleen cells from naïve mice coinjected with tumor cells and total immune splenocytes (Total + chall), $CD4^+$ (CD4 + chall) or $CD8^+$ (CD8 + chall) T cells (same animals as Fig.3A), were taken 20 days after adoptive transfer (C and D). Spleen cells were co-cultured with mitomycin C-treated parental tumor cells. Five days after stimulation, splenocytes were recovered and used as effector cells. Lactate dehydrogenase (LDH) release assay was used to measure cytotoxicity against specific tumor target cells as described in Materials and Methods. Abscissa, effector lymphocytes *versus* tumor cells (E/T) ratio; ordinate, percent of specific lysis

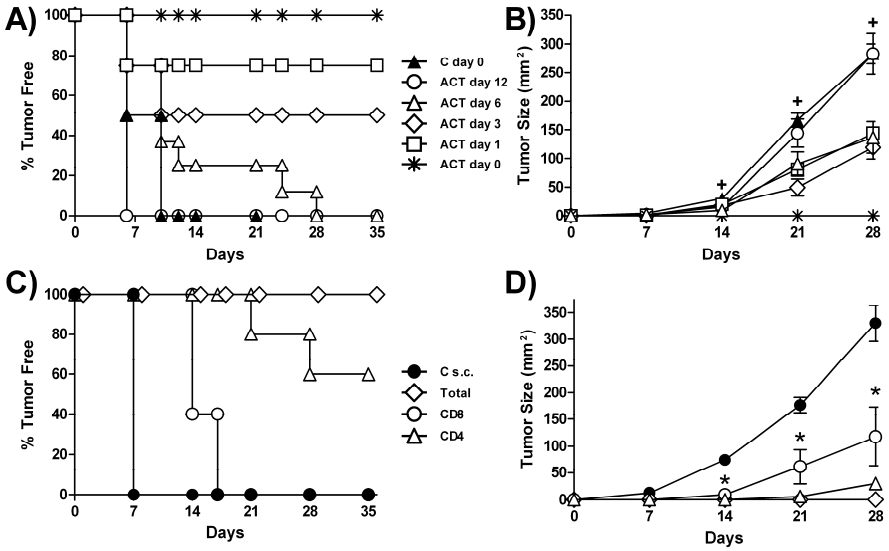


Figure 5. ACT of established tumors by CIITA-vaccine induced immune effectors.

Mice were *s.c.* injected with $5x10^4$ TS/A tumor cells. At the selected times, freshly isolated total splenocytes from mice rejecting CIITA-TS/A and a challenge with parental tumor were used for adoptive cell therapy (ACT). Immune cells were injected near the tumor site. Each mouse received $5x10^6$ total spleen cells. At least 8 animals were included per group.

A) Tumor growth was monitored over time (abscissa) and the results expressed as percentage of mice free of tumor in the ordinate.

B) Tumors still growing after ACT were compared over time (abscissa) for their corresponding size and the results expressed as tumor size (mm^2) in the ordinate. Data represent means ± SEM of tumor volumes and significant differences from parental tumor group (C day 0) are indicated (*P<0.01).

After 4-weeks from tumor challenge, a pool of spleen cells from mice with delayed tumor growth, after adoptive therapy on day 6, was used in Winn Assay experiments in which $1x10^7$ total, $3x10^6$ $CD4^+$ or $2x10^6$ $CD8^+$ cells were mixed to $5x10^4$ TS/A tumor cells. A minimum of 8 animals were included per group.

C) Results were expressed as percentage of tumor-free animals (ordinate) over time.

D) For those animals which developed a tumor, the results were expressed as tumor size (ordinate) over time (abscissa). Data represent means ± SEM of tumor volumes and significant differences from total splenocytes of not treated TS/A tumor-bearing mice control group (C sc) are indicated (*P<0.01, $^+$P<0.05).

Table 3. ACT cured mice develop long-lasting resistance to subsequent tumor challenge.

ACT group*	**Tumor free after ACT†**	**Tumor free after challenge‡**
day 0	8/8	8/8
day 1	6/8	6/6
day 3	4/8	4/4

* the listed groups include only those in which complete cure and regression of the tumor was observed.

† ratio between the number of mice undergoing ACT / the number of mice cured from tumor at 30 days after ACT.

‡ cured mice were challenged with parental tumor 30 days after first tumor injection. Results are expressed as the number of mice undergoing challenge/ the number of mice protected at 60 days after challenge

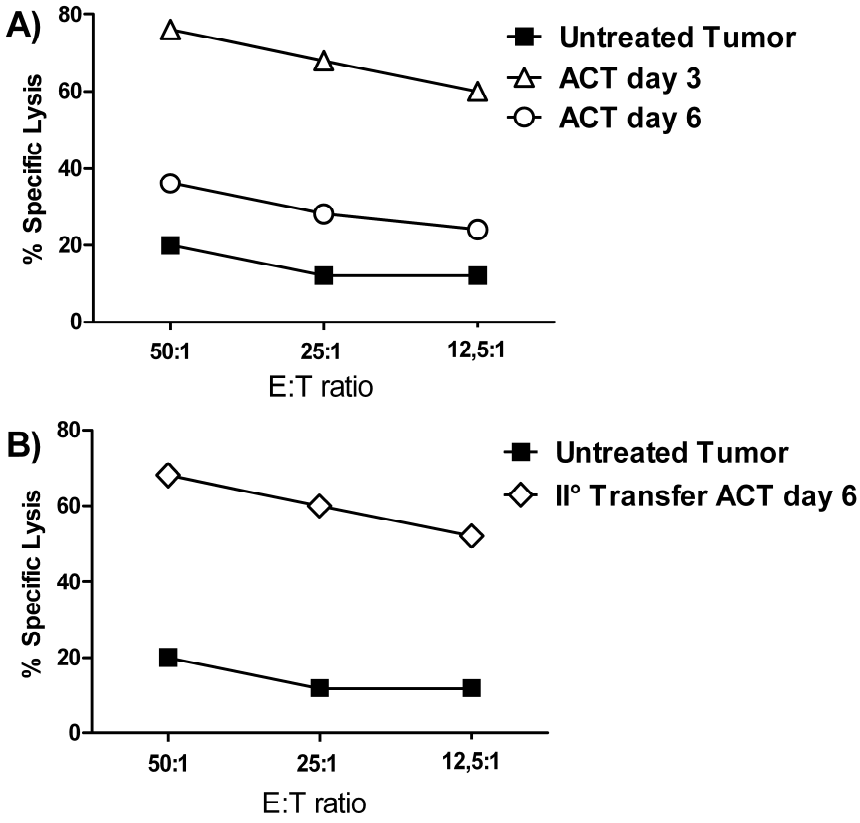


Figure 6. CTL responses in mice undergoing adoptive cell immunotherapy.

A) Splenocytes from either cured mice or mice displaying reduced growth kinetics after adoptive cell immunotherapy (ACT day 3 and ACT day 6, respectively) were assayed at after 30 days from tumor inoculum for their CTL activity.

B) Spleen cells of naïve mice that were coinjected with TS/A tumor cells and immune splenocytes from day 6 ACT and that showed no tumor growth (II° Transfer ACT day6), were assayed 30 days after coinjection for their CTL activity. Distinct splenocyte populations were incubated with mitomycin C-treated TS/A parental tumor cells. Five days after stimulation, splenocytes were used as effector cells in the Lactate dehydrogenase (LDH) release assays to measure cytotoxicity against specific tumor target cells. CTL activities were compared with the CTL activity of spleen cells from untreated, TS/A tumor bearing mice (untreated TS/A tumor). Values are expressed as percent specific lysis (ordinate) at the distinct effector/ target ratios listed in the abscissa.

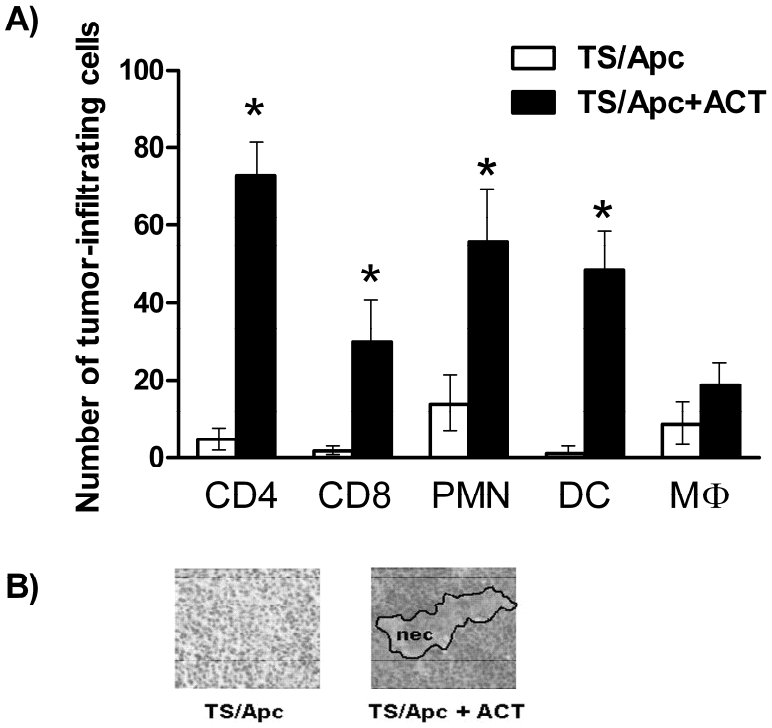


Figure 7. ACT induces a massive cellular infiltration of TS/A tumors.

Cellular infiltrates were analyzed for the presence of various leukocytes populations, namely $CD4^+$ T cells ($CD4^+$), $CD8^+$ T cells ($CD8^+$), dendritic cells (DC), polymorphonucleates (PMN), and macrophages (MΦ) in TS/A tumor tissues of mice receiving ACT at day 6 and fourteen days after tumor inoculum (A). Results are expressed as cell number (mean ± SD) per high magnification microscopic field and significant differences from control group are indicated (*P<0.01). White columns: untreated mice; black columns: mice treated with adoptive therapy on day 6. Three mice per group were used.

B), Histology of a tumor section showing extensive areas of necrosis (TS/Apc + ACT, nec) at the time of analysis after ACT as compared to tumor tissue of a control mouse injected with TS/Apc and naïve spleen cells. Staining with Gill's hematoxylin.

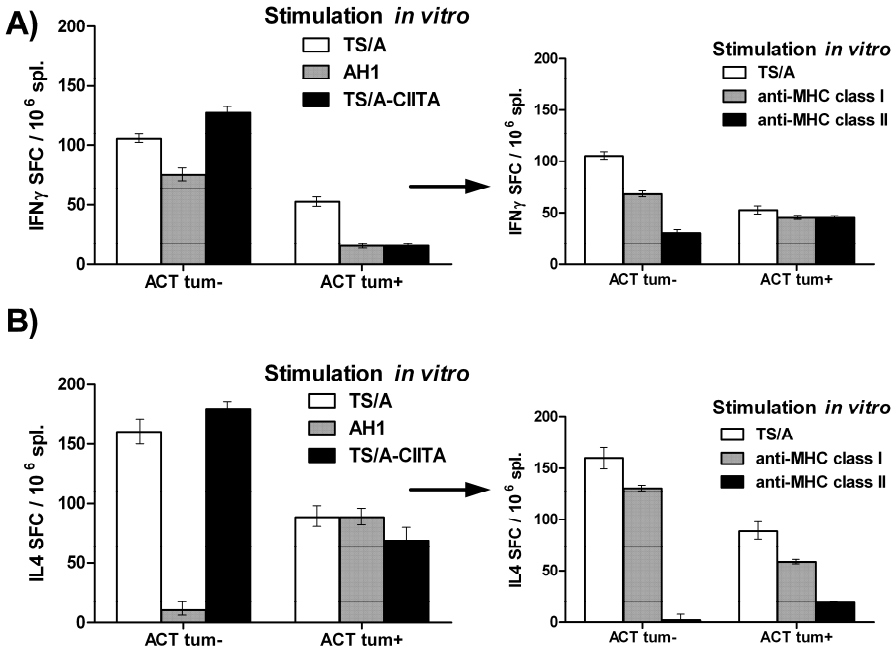


Figure 8. Th1 and Th2 responses in ACT cured or treated mice.

Tumor-specific IFN-γ- (A, left panel) and IL-4-producing (B, left panel) T cells in the spleens of mice cured (ACT tum−) or with delayed tumor growth (ACT tum^{+}) after adoptive cell immunotherapy at day 3 or at day 6, respectively. The frequencies of IFN-γ- and IL-4-producing splenocytes were evaluated by enzyme-linked immunospot assay (ELISPOT) four weeks post-tumor inoculum. The same groups of spleen cells were analyzed for IFN-γ-or IL-4-production in the presence of mAb specific for either MHC class I (anti-MHC class I) or class II (anti-MHC class II) molecules (A and B, right panels). Before ELISPOT spleen cells were restimulated *in vitro* for 40 hours with either TS/A parental cells (TS/A), TS/A-CIITA transfected cells or with the MHC-I restricted peptide AH1 from the gp70 protein of an endogenous retrovirus. Spots were detected using a stereomicroscope and values calculated per 10^6 total spleen cells. Columns, mean spot-forming cells (SFC) of triplicate samples; bars, ±SD.

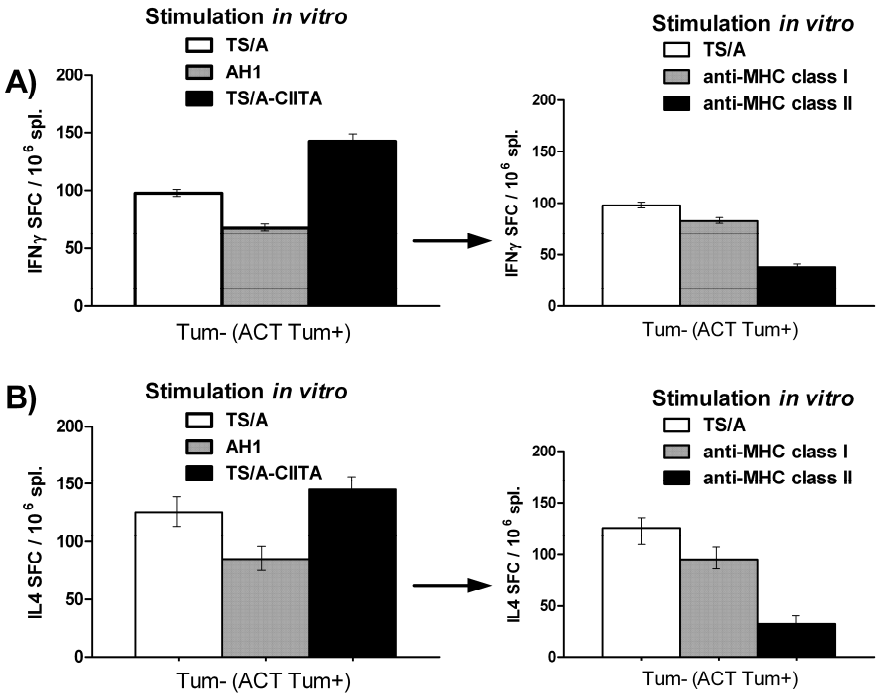


Figure 9. Th1 and Th2 responses in naïve mice after Winn Assay from ACT treated mice.

Tumor-specific IFN-γ- and IL-4-producing T cells (A and B, left panels) in the spleens of mice coinjected with TS/A and splenocytes of day 6 treated ACT animals. These animals were protected from tumor take. The assay was conducted after four weeks from coinjection. Spleen cells were also analyzed for IFN-γ-or IL-4-production in the presence of mAb specific for either MHC class I (anti-MHC class I) or class II (anti-MHC class II) molecules (A and B, right panels). Before ELISPOT spleen cells were restimulated as described in the legend to Figure 9. Spots were detected using a stereomicroscope and values calculated per 10^6 total spleen cells. Columns, mean spot-forming cells (SFC) of triplicate samples; bars, ±SD.

6. REFERENCES

Abbas and Lichtman. Cellular and Molecular Immunology, 5th ed. W. B. Saunders 2003.

Accolla RS. Human B cell variants immunoselected against a single Ia antigen subset have lost expression of several Ia antigen subsets. J Exp Med. 1983;157(3):1053-8.

Accolla RS, Scarpellino L, Carra G, Guardiola J. Trans-acting element(s) operating across species barriers positively regulate expression of major histocompatibility complex class II genes. J Exp Med. 1985;162(4):1117-33.

Accolla RS, Carra G, Guardiola J. Reactivation by a trans-acting factor of human major histocompatibility complex Ia gene expression in interspecies hybrids between an Ia-negative human B-cell variant and an Ia-positive mouse B-cell lymphoma. Proc Natl Acad Sci U S A. 1985;82(15):5145-9.

Accolla RS, Jotterand-Bellomo M, Scarpellino L, Maffei A, Carra G, Guardiola J. air-1, a newly found locus on mouse chromosome 16 encoding a trans-acting activator factor for MHC class II gene expression. J Exp Med. 1986;164(1):369-74.

Antony PA, Piccirillo CA, Akpinarli A, Finkelstein SE, Speiss PJ, Surman DR, Palmer DC, Chan CC, Klebanoff CA, Overwijk WW, Rosenberg SA, Restifo NP. $CD8^+$ 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.

Balza E, Mortara L, Sassi F, Monteghirfo S, Carnemolla B, Castellani P, Neri D, Accolla RS, Zardi L, Borsi L. Targeted delivery of tumor necrosis factor-a to tumor vessels induces a therapeutic T cell-mediated immune response that protects the host against syngeneic tumors of different histologic origin. Clin Cancer Res 2006;12:2575-82.

Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a $CD8^+$ cytotoxic T lymphocyte response by cross-priming requires cognate $CD4^+$ T cell help. J Exp Med. 1997;186(1):65-70.

Berzofsky JA, Terabe M, Oh S, Belyakov IM, Ahlers JD, Janik JE, Morris JC. Progress on new vaccine strategies for the immunotherapy and prevention of cancer. J Clin Invest. 2004;113(11):1515-25.

Bevan MJ. Helping the $CD8^+$ T-cell response. Nat Rev Immunol. 2004;4(8):595-602.

Boon T, van der Bruggen P. Human tumor antigens recognized by T lymphocytes. J Exp Med 1996;183:725-9.

Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. Annu Rev Immunol. 2006;24:175-208.

Burnet FM. The concept of immunological surveillance. Prog Exp Tumor Res. 1970;13:1-27.

Carretero R, Romero JM, Ruiz-Cabello F, Maleno I, Rodriguez F, Camacho FM, Real LM, Garrido F, Cabrera T. Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy. Immunogenetics. 2008;60(8):439-47.

Caserta S, Alessi P, Basso V, Mondino A. IL-7 is superior to IL-2 for *ex vivo* expansion of tumour-specific CD4(+) T cells. Eur J Immunol. 2009.

Cerundolo V. T cells work together to fight cancer. Curr Biol. 1999;9(18):R695-7.

Chang CH, Fontes JD, Peterlin M, Flavell RA. Class II transactivator (CIITA) is sufficient for the inducible expression of major histocompatibility complex class II genes. J Exp Med. 1994;180(4):1367-74.

Chang CH, Guerder S, Hong SC, van Ewijk W, Flavell RA. Mice lacking the MHC class II transactivator (CIITA) show tissue-specific impairment of MHC class II expression. Immunity. 1996;4(2):167-78.

Chen X, Chang CH, Goldenberg DM. Novel strategies for improved cancer vaccines. Expert Rev Vaccines 2009;8(5):567-76.

Cohen PA, Peng L, Plautz GE, Kim JA, Weng DE, Shu S. $CD4^+$ T cells in adoptive immunotherapy and the indirect mechanism of tumor rejection. Crit Rev Immunol. 2000;20:17-56.

de Waal-Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, de Vries JE. IL-10 and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down-regulation of class II MHC expression. J Exp Med 1991;174:915.

Dolan BP, Gibbs KD Jr, Ostrand-Rosenberg S. Tumor-specific $CD4^+$ T cells are activated by "cross-dressed" dendritic cells presenting peptide-MHC class II complexes acquired from cell-based cancer vaccines. J Immunol. 2006;176(3):1447-55.

Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 2002;298:850-4.

Dudley ME and Rosenberg SA. Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat. Rev. Cancer. 2003;3:666–675.

Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, Rogers LJ, Gracia GJ, Jones SA, Mangiameli DP, Pelletier MM, Gea-Banacloche J, Robinson MR, Berman DM, Filie AC, Abati A, Rosenberg SA. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol. 2005;23(10):2346-57.

Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, Robbins PF, Huang J, Citrin DE, Leitman SF, Wunderlich J, Restifo NP, Thomasian A, Downey SG, Smith FO, Klapper J, Morton K, Laurencot C, White DE, Rosenberg SA. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J Clin Oncol. 2008;26(32):5233-9.

Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol. 2004;22:329-60.

Festenstein H, Garrido F. MHC antigens and malignancy. Nature. 1986;322(6079):502-3.

Finke J, Ferrone S, Frey A, Mufson A, Ochoa A. Where have all the T cells gone? Mechanisms of immune evasion by tumors. Immunol Today. 1999;20(4):158-60.

Garrido F, Ruiz-Cabello F, Cabrera T, Pérez-Villar JJ, López-Botet M, Duggan-Keen M, Stern PL. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol Today. 1997;18(2):89-95.

Gattinoni L, Finkelstein SE, Klebanoff CA, Antony PA, Palmer DC, Spiess PJ, Hwang LN, Yu Z, Wrzesinski C, Heimann DM, Surh CD, Rosenberg SA, Restifo NP. Removal of homeostatic cytokine

sinks by lymphodepletion enhances the efficacy of adoptively transferred tumour-specific $CD8^+$ T cells. J Exp Med 2005;202:907–912.

Gattinoni L, Powell DJ Jr, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol. 2006;6(5):383–393.

Hahne M, Rimoldi D, Schröter M, Romero P, Schreier M, French LE, Schneider P, Bornand T, Fontana A, Lienard D, Cerottini J, Tschopp J. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. Science 1996;274(5291):1363-6.

Hamilton SE, Wolkers MC, Schoenberger SP, Jameson SC. The generation of protective memory-like $CD8^+$ T cells during homeostatic proliferation requires $CD4^+$ T cells. Nat Immunol. 2006;7(5):475-81.

Hanahan D and Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.

Huang AY, Gulden PH, Woods AS, Thomas MC, Tong CD, Wang W, Engelhard VH, Pasternack G, Cotter R, Hunt D, Pardoll DM, Jaffee EM. The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon derives from an endogenous retroviral gene product. Proc. Natl. Acad. Sci. USA 1996;93: 9730–9735.

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.

Jager D, Jager E, Knuth A. Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer. J Clin Pathol 2001;54:669–74.

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.

Jorritsma A, Bins AD, Schumacher TN, Haanen JB. Skewing the T-cell repertoire by combined DNA vaccination, host conditioning, and adoptive transfer. Cancer Res. 2008;68(7):2455-62.

Kennedy R and Celis E. Multiple roles for $CD4^+$ T cells in anti-tumor immune responses. Immunol Rev 2008;222:129-44.

Kern I, Steimle V, Siegrist CA, Mach B. The two novel MHC class II transactivators RFX5 and CIITA both control expression of HLA-DM genes. Int Immunol. 1995;7(8):1295-9.

Kim D, Hoory T, Monie A, Ting JPY, Hung CF, Wu TC. Enhancement of DNA vaccine potency through coadministration of CIITA DNA with DNA vaccines via gene gun. J Immunol 2008;180:7019-27.

Klebanoff CA, Khong HT, Antony PA, Palmer DC, Restifo NP. Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumour immunotherapy. Trends Immunol 2005;26:111–117.

Kruger-Krasagakes S, Krasagakis K, Garbe C, Schmitt E, Hüls C, Blankenstein T, Diamantstein T. Expression of interleukin 10 in human melanoma. Br J Cancer. 1994;70:1182–1185.

Kwok RP, Lundblad JR, Chrivia JC, Richards JP, Bächinger HP, Brennan RG, Roberts SG, Green MR, Goodman RH. Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature. 1994;370:223-6.

Lai P, Rabinowich H, Crowley-Nowick PA, Bell MC, Mantovani G, Whiteside TL. Alterations in expression and function of signal-transducing proteins in tumor-associated T and natural killer cells in patients with ovarian carcinoma. Clin Cancer Res. 1996;2(1):161-73.

Levey DL, Srivastava PK. Alterations in T cells of cancer-bearers: whence specificity? Immunol Today. 1996;17(8):365-8.

Lopez MV, Adris SK, Bravo AI, Chernajovsky Y, Podhajcer OL. IL-12 and IL-10 expression synergize to induce the immune-mediated eradication of established colon and mammary tumors and lung metastasis. J Immunol. 2005;175(9):5885-94.

Lou Y, Wang G, Lizée G, Kim GJ, Finkelstein SE, Feng C, Restifo NP, Hwu P. Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells *in vivo*. Cancer Res. 2004;64(18):6783-90.

Maeurer MJ, Gollin SM, Martin D, Swaney W, Bryant J, Castelli C, Robbins P, Parmiani G, Storkus WJ, Lotze MT. Tumor escape from immune recognition: lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen. J Clin Invest. 1996;98(7):1633-41.

Matsuda M, Salazar F, Petersson M, Masucci G, Hansson J, Pisa P, Zhang QJ, Masucci M, Kiessling R. Interleukin-10 pre-treatment protects target cells from tumor- and allo-specific cytotoxic T-cells and down-regulates HLA class I expression. J Exp Med 1994;180:2371.

Matsuda M, Petersson M, Lenkei R, Taupin JL, Magnusson I, Mellstedt H, Anderson P, Kiessling R. Alterations in the signal-transducing molecules of T cells and NK cells in colorectal tumor-infiltrating, gut mucosal and peripheral lymphocytes: correlation with the stage of the disease. Int J Cancer. 1995;61(6):765-72.

Meazza R, Comes A, Orengo AM, Ferrini S, Accolla RS. Tumor rejection by gene transfer of the MHC class II transactivator in murine mammary adenocarcinoma cells. Eur J Immunol. 2003;33(5):1183-92.

Mizoguchi H, O'Shea JJ, Longo DL, Loeffler CM, McVicar DW, Ochoa AC. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. Science 1992;258:1795.

Moeller M, Haynes NM, Kershaw MH, Jackson JT, Teng MW, Street SE, Cerutti L, Jane SM, Trapani JA, Smyth MJ, Darcy PK. Adoptive transfer of gene-engineered $CD4^+$ helper T cells induces potent primary and secondary tumor rejection. Blood. 2005;106(9):2995-3003.

Mortara L, Castellani P, Meazza R, Tosi G, De Lerma Barbaro A, Procopio FA, Comes A, Zardi L, Ferrini S, Accolla RS. CIITA-induced MHC class II expression in mammary adenocarcinoma leads to a Th1 polarization of the tumor microenvironment, tumor rejection, and specific antitumor memory. Clin Cancer Res. 2006;12(11 Pt 1):3435-43.

Mortara L, Balza E, Sassi F, Castellani P, Carnemolla B, De Lerma Barbaro A, Fossati S, Tosi G, Accolla RS, Borsi L. Therapy-induced antitumor vaccination by targeting tumor necrosis factor alpha to tumor vessels in combination with melphalan. Eur J Immunol. 2007;37(12):3381-92.

Mortara L, Frangione V, Castellani P, De Lerma Barbaro A, Accolla RS. Irradiated CIITA-positive mammary adenocarcinoma cells act as a potent anti-tumor-preventive vaccine by inducing tumor-specific $CD4^+$ T cell priming and $CD8^+$ T cell effector functions. Int Immunol 2009;21:655-65.

Moutsopoulos NM, Wen J, Wahl SM. TGF-beta and tumors--an ill-fated alliance. Curr Opin Immunol. 2008;20(2):234-40.

Muhlethaler-Mottet A, Di Berardino W, Otten LA, Mach B. Activation of the MHC class II transactivator CIITA by interferon-gamma requires cooperative interaction between Stat1 and USF-1. Immunity. 1998;8(2):157-66.

Muranski P, Restifo NP. Adoptive immunotherapy of cancer using $CD4(^+)$ T cells. Curr Opin Immunol 2009;21:200-8.

Nakagomi H, Petersson M, Magnusson I, Juhlin C, Matsuda M, Mellstedt H, Taupin JL, Vivier E, Anderson P, Kiessling R. Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma. Cancer Res. 1993;53(23):5610-2.

Nanni P, De Giovanni C, Lollini PL, Nicoletti G, Prodi G. TS/A: a new metastasizing cell line from a BALB/c spontaneous mammary adenocarcinoma. Clin Exp Metastasis 1983;1:373-80.

Nishimura T, Iwakabe K, Sekimoto M, Ohmi Y, Yahata T, Nakui M, Sato T, Habu S, Tashiro H, Sato M, Ohta A. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication *in vivo*. J Exp Med. 1999;190(5):617-27.

Ochsenbein AF, Sierro S, Odermatt B, Pericin M, Karrer U, Hermans J, Hemmi S, Hengartner H, Zinkernagel RM. Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. Nature. 2001;28;411(6841):1058-64.

Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, Dellemijn TA, Antony PA, Spiess PJ, Palmer DC, Heimann DM, Klebanoff CA, Yu Z, Hwang LN, Feigenbaum L, Kruisbeek AM, Rosenberg SA, Restifo NP. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive $CD8^+$ T cells. J Exp Med. 2003;198(4):569-80.

Pardoll DM, Topalian SL. The role of $CD4^+$ T cell responses in antitumor immunity. Curr Opin Immunol. 1998;10(5):588-94.

Pardoll D. T cells and tumours. Nature 2001;411(6841):1010-2.

Parviz M, Chin CS, Graham LJ, Miller C, Lee C, George K, Bear HD. Successful adoptive immunotherapy with vaccine-sensitized T cells, despite no effect with vaccination alone in a weakly immunogenic tumor model. Cancer Immunol Immunother. 2003;52(12):739-50.

Peng Y, Laouar Y, Li MO, Green EA, Flavell RA. TGF-beta regulates *in vivo* expansion of Foxp3-expressing $CD4^+CD25^+$ regulatory T cells responsible for protection against diabetes. Proc Natl Acad Sci USA. 2004;101:4572–4577.

Perez-Diez A, Joncker NT, Choi K, Chan WF, Anderson CC, Lantz O, Matzinger P. CD4 cells can be more efficient at tumor rejection than CD8 cells. Blood. 2007;109:5346-54.

Piskurich JF, Wang Y, Linhoff MW, White LC, Ting JP. Identification of distinct regions of 5' flanking DNA that mediate constitutive, IFN-gamma, STAT1, and TGF-beta-regulated expression of the class II transactivator gene. J Immunol. 1998;160(1):233-40.

Piskurich JF, Linhoff MW, Wang Y, Ting JP. Two distinct gamma interferon-inducible promoters of the major histocompatibility complex class II transactivator gene are differentially regulated by STAT1, interferon regulatory factor 1, and transforming growth factor beta. Mol Cell Biol. 1999;19(1):431-40.

Ploegh HL. Viral strategies of immune evasion. Science. 1998;280:248–253.

Rosenberg SA and Dudley ME. Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. Proc Natl Acad Sci U S A. 2004;101 Suppl 2:14639-45.

Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med. 2004;10(9):909-15.

Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell therapy: a clinical path to effective cancer immunotherapy. Nat Rev Cancer 2008;8:299-308.

Salazar-Onfray F, Charo J, Petersson M, Freland S, Noffz G, Qin Z, Blankenstein T, Ljunggren HG, Kiessling R. Down-regulation of the expression and function of the transporter associated with antigen processing in murine tumor cell lines expressing IL-10. J Immunol. 1997;159(7):3195-202.

Sartoris S, Tosi G, De Lerma Barbaro A, Cestari T, Accolla RS. Active suppression of the class II transactivator-encoding AIR-1 locus is responsible for the lack of major histocompatibility complex class II gene expression observed during differentiation from B cells to plasma cells. Eur J Immunol. 1996;26(10):2456-60.

Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 1998;393(6684):480-3.

Schuler T, Qin Z, Ibe S, Noben-Trauth N, Blankenstein T. T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice. J Exp Med 1999;189:803-10.

Song E, Chen J, Ouyang N, Su F, Wang M, Heemann U. Soluble Fas ligand released by colon adenocarcinoma cells induces host lymphocyte apoptosis: an active mode of immune evasion in colon cancer. Br J Cancer 2001;85:1047–54.

Steimle V, Otten LA, Zufferey M, Mach B. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). Cell. 1993;75(1):135-46.

Steimle V, Siegrist CA, Mottet A, Lisowska-Grospierre B, Mach B. Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. Science. 1994;265(5168):106-9.

Steinbrink K, Jonuleit H, Müller G, Schuler G, Knop J, Enk AH. Interleukin-10-treated human dendritic cells induce a melanoma-antigen- specific anergy in CD8($^{+}$) T cells resulting in a failure to lyse tumor cells. Blood. 1999;93:1634–1642.

Tamai H, Watanabe S, Zheng R, Deguchi K, Cohen PA, Koski GK, Shu S. Effective treatment of spontaneous metastases derived from a poorly immunogenic murine mammary carcinoma by combined dendritic-tumor hybrid vaccination and adoptive transfer of sensitized T cells. Clin Immunol. 2008;127(1):66-77.

Tanaka H, Yoshizawa H, Yamaguchi Y, Ito K, Kagamu H, Suzuki E, Gejyo F, Hamada H, Arakawa M. Successful adoptive immunotherapy of murine poorly immunogenic tumor with specific effector cells generated from gene-modified tumor-primed lymph node cells. J Immunol. 1999;162(6):3574-82.

Tartour E, Latour S, Mathiot C, Thiounn N, Mosseri V, Joyeux I, D'Enghien CD, Lee R, Debre B, Fridman WH. Variable expression of CD3-zeta chain in tumor-infiltrating lymphocytes (TIL) derived from renal-cell carcinoma: relationship with TIL phenotype and function. Int J Cancer. 1995;63(2):205-12.

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst. 2000;92(3):205-16.

van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, van den Eynde BJ, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991;254:1643-7.

Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nat Med. 2004;10(8):789-99.

Whiteside TL. Signaling defects in T lymphocytes of patients with malignancy. Cancer Immunol Immunother. 1999;48(7):346-52.

Winter H, Hu HM, Poehlein CH, Huntzicker E, Osterholzer JJ, Bashy J, Lashley D, Lowe B, Yamada J, Alvord G, Urba WJ, Fox BA. Tumour-induced polarization of tumour vaccine-draining lymph node T cells to a type 1 cytokine profile predicts inherent strong immunogenicity of the tumour and correlates with therapeutic efficacy in adoptive transfer studies. Immunology. 2003;108(3):409-19.

Wittke F, Hoffmann R, Buer J, et al. Interleukin 10 (IL-10): an immunosuppressive factor and independent predictor in patients with metastatic renal cell carcinoma. Br J Cancer. 1999;79:1182–1184.

Wright KL, Ting JP. Epigenetic regulation of MHC CLASS II and CIITA genes. Trends Immunol. 2006;27(9):405-12.

Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, Greenberg PD. Adoptive T cell therapy using antigen-specific $CD8^+$ T cell clones for the treatment of patients with metastatic melanoma: *in vivo* persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A. 2002;99(25):16168-73.

Yewdell JW, Bennink JR. Mechanisms of viral interference with MHC class I antigen processing and presentation. Annu Rev Cell Dev Biol. 1999;15:579–606.

Zea AH, Curti BD, Longo DL, Alvord WG, Strobl SL, Mizoguchi H, Creekmore SP, O'Shea JJ, Powers GC, Urba WJ, Ochoa AC. Alterations in T cell receptor and signal transduction molecules in melanoma patients. Clin Cancer Res. 1995;1(11):1327-35.

Zhu XS, Linhoff MW, Li G, Chin KC, Maity SN, Ting JP. Transcriptional scaffold: CIITA interacts with NF-Y, RFX, and CREB to cause stereospecific regulation of the class II major histocompatibility complex promoter. Mol Cell Biol. 2000;20(16):6051-61.

Zika E, Ting JPY. Epigenetic control of MHC CLASS II: interplay between CIITA and histone-modifying enzymes. Curr. Opin. Immunol. 2005;17:58-64.