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**Class II Transactivator-induced MHC class II expression in pancreatic cancer cells leads to tumor rejection and a specific antitumor memory response**

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**Running title:** *De novo* expression of MHC II elicits rejection of PDA

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## Abstract

**Objectives:** The loss of major **histocompatibility complex (MHC)** classes I and II is a well-known mechanism by which cancer cells are able to escape from immune recognition. In this study, we analyzed the expression of antigen processing and presenting molecules in two cell lines derived from mouse models of pancreatic ductal adenocarcinoma (PDA), and the effects of the re-expression of MHC class II on PDA rejection.

**Methods:** PDA cell lines were analysed for the expression of MHC class I, II and antigen-processing molecules by flow cytometry or **polymerase chain reaction**. We generated stable PDA-MHC class II Transactivator (CIITA) cells and injected them into syngeneic mice. CD4 and CD8 T cell role was analysed *in vitro* and *in vivo*.

**Results:** Murine PDA cell lines were negative for MHC and antigen-processing molecules, but their expression was restored by exogenous interferon- $\gamma$ . Tumor cells were rejected in 80% to 100% of injected mice, which also developed long-lasting immune memory. *In vitro* assays and immunohistochemical analyses revealed **the recruitment of T effector cells and CD8 T cells into the tumor area**.

**Conclusions:** **Overall, these data confirm that immunotherapy is a feasible therapeutical approach in order to recognize and target an aggressive cancer such as PDA.**

**Key words:** pancreatic cancer, immunotherapy, AIR-1 locus-encoded MHC class II transactivator, MHC class I and II molecules, T cell.

## Introduction

Tumor immune escape plays a critical role in cancer, but the mechanisms involved in this process are not yet well defined. In recent years, progress has been made in understanding how peptides presented by MHC class I molecules are generated, in particular which proteases are involved in this process, and how intracellular pathways influence antigen presentation in professional antigen-presenting cells and in various types of malignancies. Different MHC class I abnormalities have been found in solid tumors of distinct origin but also in hematopoietic diseases <sup>1,2</sup>. Changes include structural alterations such as total, haplotype and allelic loss of the MHC class I heavy chain; deletions and point mutations, in particular in *beta 2*-microglobulin and TAP1 (transporter for antigen presentation-1), as well as dysregulation of various components of the MHC class I antigen processing machinery (APM), which can occur at the epigenetic, transcriptional or post-transcriptional level <sup>3</sup>. The down-modulation or lack of expression of single or multiple components of the MHC class I antigen processing pathway may prevent the recognition and killing of tumor cells by tumor-specific CD8 cytotoxic T lymphocytes (CTL). In a murine tumor model, loss of TAP was associated with increased tumorigenesis and tumor growth advantage compared to normal TAP-expressing counterparts <sup>4</sup>. However, this loss is reversed by exogenous interferon treatment, suggesting a regulatory rather than a structural defect in TAP expression <sup>5</sup>.

Several attempts have been made to modify the antitumor immune response and the tumor environment, either by tumor-specific MHC class I-restricted peptide vaccination to rescue CTL activity, or by increasing the availability of soluble mediators, including cytokines and chemokines <sup>6</sup>. However, CTL responses were generally weak and unable to control tumor growth in most patients when CTL-defined antigens were administered as vaccines in clinical trials <sup>7</sup>. It is now clear that this is mostly due to low numbers of tumor-specific, MHC class II-restricted CD4 T helper cells being generated in tumor-bearing patients <sup>8</sup>. In fact, T helper (Th) cells are required for optimal induction of both humoral and cellular effector mechanisms <sup>9,10</sup>, and CD40L- mostly expressed by Th cells or by CD8 T cells <sup>11</sup>- is necessary for inducing CTL cross-priming by

dendritic cells<sup>8</sup>. Most attempts at producing relevant Th-derived cytokines and other soluble mediators in the tumor microenvironment have been mainly carried out by genetic transfer of relevant cDNAs into tumor cells. Diverse cytokines and chemokines may lead to tumor regression both by direct anti-angiogenic effects, as in the case of interferon (IFN)- $\gamma$ <sup>12</sup>, and by activation of anti-tumor immunity<sup>13, 14</sup>. Nevertheless, clinical application is seriously impeded by the difficulty in controlling efficacy in terms of amount and duration, and the possible adverse effects generated by the release of certain cytokines. In addition, some studies have been focused on creating second generation peptides, i.e. multiple, personalized and hybrid peptides consisting of CTL and Th epitopes<sup>15</sup>. The use of professional antigen presenting cells (APC), such as dendritic cells (DC) pulsed with tumor antigen peptides *in vitro* and re-infused *in vivo*, has recently been improved by the use of “third-generation” DC with an elevated rather than an “exhausted” ability to produce activating factors for Th1, CTL and NK cells<sup>16</sup>. Clinical trials with the first two generations of DC showed limited clinical responses, while those using the third-generation DC are still ongoing in order to demonstrate efficacy on overall survival. An alternative therapeutic possibility would be to render the tumor cells themselves surrogate APCs by inducing them to express the MHC class II molecules, which bind antigenic peptides and present them to CD4 T cells. One clear example of this was the murine TS/A mammary adenocarcinoma cells transfected with the AIR-1 locus-encoded MHC class II transactivator, CIITA<sup>17-19</sup>, and thus stably expressing MHC class II molecules. *In vivo* challenge resulted in 50% of tumor cells being rejected, due to the elicited activation of both CD4 Th and CD8 CTL cells<sup>18</sup>.

PDA cell lines, or tissues from patients, express very low levels of MHC class I and II molecules, or indeed may totally lack them<sup>20-22</sup>. In this study, we analyzed the processing and presenting components in murine PDA cell lines obtained from an endogenous mouse model, and the *in vivo* effects on PDA growth of the re-expression of MHC class II molecules due to transfection with CIITA. We show that stable expression of CIITA in PDA cells results in complete tumor rejection *in vivo*, along with the development of a well-established anti-tumor memory against parental tumor

cells. We thus postulate that PDA-CIITA cells process and present nominal antigens to CD4 T cells, supported by the fact that strong tumor infiltration by both CD4 and CD8 T cells was observed. Therefore, these data highlight the need for adequate activation of both CD4 Th and CD8 CTL cells for a successful therapeutic strategy, which may also be advantageous in the case of an aggressive tumor such as PDA. A favorable inflammatory response, stimulated by different kinds of treatment, may create the most suitable cytokine environment for inducing the re-expression of both MHC class I and class II molecules.



## **Materials and methods:**

### **Cell lines.**

DT6606 and K8484 cells are murine pancreatic cancer cell lines obtained from mice carrying single mutated Kras<sup>G12D</sup> or double mutated Kras<sup>G12D</sup> and Trp53<sup>R172H</sup> (kindly provided by Dr. K. Olive, Columbia University). Cell lines were maintained in Dulbecco's medium (Sigma–Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (Life Technologies, Milan, Italy), 2 mM glutamine (Sigma–Aldrich), and antibiotics. In some experiments, cell lines were treated with 100 U/ml IFN- $\gamma$  (Sigma–Aldrich) for 48 h.

### **Analysis of MHC-I and II surface expression.**

MHC class I and class II surface expression was analyzed by immunofluorescence using a FACSCalibur (BD Biosciences, Milan, Italy) according to a standard protocol. Primary antibodies used were purified anti-H-2K<sup>b</sup> (20-8-4S) and anti-H-2D<sup>b</sup> (28.14.8) (ATCC (Rockville, MD, USA) followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse (Sigma-Aldrich), and a phycoerythrin (PE)-conjugated anti-I-A/E (clone M5/14.15.2, Biolegend). Isotype-matched non-immune rat IgG and cells labeled only with the FITC-conjugated antibody were used as controls. A minimum of  $1 \times 10^4$  cells was analyzed with Cell-Quest Pro software (BD Biosciences). Each cell line was first tested after only one culture passage, and then re-tested at least three times more during different passages. All cell lines were analyzed in basal conditions and after treatment with IFN- $\gamma$  (100 U/ml) for 48 h.

### **Real time RT–PCR.**

An *mRNA isolation kit* (Miltenyi-Biotech, GmbH Germany) was used to extract mRNA from DT6606 and K8484 cell lines. First-strand cDNA was synthesized with 100 ng of mRNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a total volume

of 20  $\mu$ l. These cDNAs were diluted to a final volume of 100  $\mu$ l. Real-time quantitative RT-PCR analyses for  $\beta$ 2-microglobulin, H2-K<sup>b</sup>, H2-D<sup>b</sup>, TAP1, TAP2, LMP2, LMP7, calnexin, calreticulin, and tapasin genes were performed in the 7500 Fast System (Applied Biosystems), using *Gapdh* and  *$\beta$ -actin* genes as housekeeping genes. PCR reactions were performed in quadruplicate, and values obtained were expressed as means  $\pm$  SD (standard deviation). Real-time quantitative RT-PCR was performed with the Power SYBR Green Master mix (Applied Biosystems). Primers and amplicon size for each gene were previously described<sup>23</sup>. PCR conditions were as follows: 40 cycles of 15s of denaturation at 95°C and 60s at 60°C.

### **Transfection of CIITA.**

Transfection of CIITA plasmid was cloned in the *Eco*RI site of the pcDNA3.1 vector (Invitrogen, Milan, Italy). fCIITA pcDNA3 vector coding for FLAG-tagged full-length CIITA<sup>1-1130</sup> was used to transfect K8484 and DT6606 cells with the Effectene Transfection Reagent (Qiagen, Milan, Italy). Transfected cells were selected with 1 mg/ml G-418 (Sigma-Aldrich). Highly positive MHC class II cells were purified by cell sorting (MoFlow, DakoCytomation, Milan, Italy). Antisense preparation of pcDNA3.1-CIITA plasmid was obtained by digestion with BamHI (Fermentas by M-Medical, Milan, Italy), gel purification of fragments and re-ligation into the same vector in reverse orientation.

### **In vivo immunization.**

Female C57BL/6 mice of 5 weeks old were injected subcutaneously (s.c.) with  $1 \times 10^5$  K8484-CIITA or DT6606-CIITA-transfected tumor cells or the relative cells transfected with the antisense sequence as a control. Tumor diameters were measured biweekly using a caliper. For *in vivo* immune memory experiments, mice were injected with parental PDA-derived K8484 and DT6606 cells in the contro-lateral flank after 3 weeks from the initial PDA-CIITA cell challenge. All mice were treated following European Guidelines and sacrificed when signs of suffering appeared.

### **CTL activity assay.**

A commercially-available lactate dehydrogenase (LDH) kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega, Milan, Italy) was used with parental K8484 and DT6606 as the target cells. Briefly, effector cells from either CIITA-injected or CIITA-antisense-injected mice were mixed with target cells at different ratios (1:12, 1:25, and 1:50) in a 96-well U-bottomed plate and incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After incubation, the plate was centrifuged; supernatants were collected and incubated for 30 min at room temperature, followed by detection of LDH activity using the substrate mix provided with the kit. A stop solution was added and the absorbance read at 490 nm with an Elisa microplate reader (BioRad, Milan, Italy). All determinations were performed in triplicate. The percentage of cell-mediated cytotoxicity was calculated by subtracting the spontaneous LDH released by target and effector cells from the LDH released by lysed target cells, using the following equation:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100.$$

### **Immunohistochemistry.**

Paraffin-embedded sections were de-paraffinized and rehydrated in different series of alcohol. They were then blocked with 3% H<sub>2</sub>O<sub>2</sub> for 10 min, followed with antigen retrieval in citrate buffer (Dako) in a microwave oven. Sections were washed and incubated overnight at 4°C with monoclonal antibodies against CD4 (clone YTS 191.1.2; ImmunoTools, Friesoythe, Germany) and CD8 (clone 53-6.7; eBioscience by Prodotti Gianni, Milan, Italy) at an optimal dilution of 1:100 in DAKO antibody dilution buffer (Dako). After washing, they were further incubated with anti-rat HRP secondary antibody for 30 min at room temperature. 3,3'-diaminobenzidine tetrahydrochloride (Vector, DBA, Milan, Italy) was used as the chromogen and hematoxylin (Dako) to counterstain tissues. Sections were covered with slides with Bio-Mount medium (Bio-Optica, Milan, Italy).



## Results

### *MHC class I surface expression*

MHC class I cell surface expression on DT6606 and K8484 murine tumor cells was analyzed by flow cytometry. Both cell lines are characterized by negative H-2K<sup>b</sup> and H-2D<sup>b</sup> surface expression in basal conditions, but both H-2 class I molecules were upregulated after IFN- $\gamma$  treatment (Fig. 1A). To analyze the mechanisms underlying the basal loss of MHC class I molecules in these PDA cells, the mRNA expression of H-2 class I heavy chains,  $\beta$ 2-microglobulin, and several APM components, was analyzed by real-time RT-PCR, and compared with a positive MHC class I cell line (TC-1). Data were normalized using  $\beta$ -actin and *Gapdh* as housekeeping genes. Figure 1B shows the mRNA expression normalized against reference TC-1 values (relative value of 1). Both PDA cell lines display similar expression patterns, i.e. a strong down-regulation of all analyzed genes, but not the complete loss of H-2D and H-2K genes, which suggests a post-transcriptional regulation. In addition, we found a total absence of transcription of the LMP-2 and LMP-7 proteasome components in both cells lines, suggesting the absence of immunoproteasome. In these MHC class I negative cell lines, treatment with IFN- $\gamma$  increased the transcriptional levels of several analyzed genes (data not shown). These results demonstrate a direct relationship between the transcriptional level of MHC class I-related genes and MHC class I surface expression in these PDA cell lines.

### *CIITA gene transfection in poorly expressing MHC class I PDA cells induces tumor rejection and a memory immune response.*

Transfection of CIITA in poorly immunogenic PDA cells resulted in increased expression levels of the MHC class II molecule, as observed by flow cytometry (Fig. 2). The *in vitro* morphology and growth kinetics of CIITA-transfected cells did not differ compared to untransfected or CIITA-antisense transfected cells (data not shown). When CIITA-transfected tumor cells were injected into

syngeneic mice, complete rejection or significant delay in tumor growth was observed (Fig. 3A, B). Specifically, complete rejection was observed in 80% of mice injected with CIITA-K8484 cells, while in the remaining 20%, CIITA-K8484 cells grew significantly slower compare to antisense CIITA-K8484 cells. All mice injected with CIITA-DT6606 cells demonstrated complete tumor rejection after 20 days, while those injected with antisense CIITA-DT6606 developed tumors of up to 10 mm in diameter by 30 days. The antisense CIITA-transfected DT6606 cells grew in a similar manner to the untransfected parental tumors.

We then evaluated whether immunization with CIITA-expressing cells elicited a specific memory response against parental PDA cells. Mice were challenged with parental cells, in the opposite flank, 24 days after CIITA-transfected PDA cell injection. All mice that had been immunized with sense CIITA K8484 and DT6606 cells showed complete rejection of parental PDA cells compared to those injected with antisense CIITA-transfected cells (Fig. 3C, D). These results demonstrate that immunization with CIITA-mediated MHC class II-expressing PDA cells results in long-lasting, specific antitumor immunity against MHC I- and II-negative parental tumor cells.

***Expression of CIITA-dependent MHC class II elicits a specific cytotoxic response by both CD4 and CD8 cells.***

Due to the rapid rejection of parental PDA cells after immunization with CIITA-transfected cells, we evaluated the effector role of CD4 and CD8 cells *in vitro*. T lymphocytes were isolated from spleens of immunized mice, and analyzed, by means of the LDH non-radioactive cytotoxic assay, for the ability to lyse parental tumor cells. CTLs from mice immunized with CIITA-transfected K8484 or DT6606 efficiently mediated up to 25–30% more lysis compared to splenocytes obtained from antisense CIITA-transfected cells (Fig. 4). The difference in lysis was significant at all effector: target (E:T) ratios tested ( $P < 0.005$ ). To determine whether the lysis was MHC class I or class II restricted, mAb directed against H-2K<sup>b</sup>/H-2D<sup>b</sup> MHC class I and I-A/I-E MHC class II were

added to target cells. Their presence abrogated cytotoxic activity by up to 40-50%. When both antibodies were added together, cytotoxic activity was almost entirely inhibited (Fig. 4).

***CIITA-expressing PDA cells significantly recruit both CD4 and CD8 T cells into the tumor area.***

To establish the presence of effector cells recruited from CIITA-transfected K8484 and DT6606 PDA cells, tumor tissues were analyzed by immunohistochemistry. Of particular note, extensive areas of sense CIITA-transfected tumors show a high number of CD4 T cells (Fig. 5A, C) compared to masses from antisense CIITA-transfected PDA cells (Fig. 5E, G). A similar flux of cells was observed when tumor tissues were stained for CD8 antigen (Fig. 5B, D). Antisense CIITA-transfected cells (Fig. 5F, H), as well as parental cells (data not shown), did not present CD8 T cell infiltration. These data support the correlation between the presence of both CD4 and CD8 T cells and tumor rejection of sense CIITA-transfected tumors. Indeed, the absence of MHC class II expression did not elicit such a favorable inflammatory environment for recruiting T cells, as demonstrated by the absence of CD4 and CD8 T cells in antisense CIITA-transfected tumors.

## Discussion

PDA is a tremendously disabling, dismal disease, of which surgical resection is currently the only chance of cure. Unfortunately, this is only possible in 20% of newly-diagnosed patients as it is still impossible to make an early diagnosis. Thus, novel therapeutical approaches are urgently needed. In the current study, we demonstrate that the re-expression of MHC class II from poorly immunogenic PDA cells elicits a strong immune reaction able to reject transplantable tumors and establish a long-lasting immune memory.

Understanding the dual role of the immune system on cancer cells has strongly revitalized efforts from the scientific community to design novel and more efficient therapeutical strategies<sup>24</sup>. Similar efforts have been exerted for the identification of novel tumor-associated antigens through different approaches. Vaccines and immunotherapy have, therefore, gained additional importance in recent years, thanks to monumental achievements in murine and clinical studies. In particular, successful vaccination confers multiple immune effector arms, including the generation of CD4 and CD8 T cells, as well as the combination with factors that limit the immunosuppression exerted by the immune system, in order to generate a potent anti-tumor response<sup>10</sup>.

We have recently demonstrated that a DNA vaccine against a novel PDA-associated antigen, namely alpha-enolase (ENO1)<sup>25</sup>, significantly prolongs median survival in a PDA mouse model by inducing an integrated humoral and cellular immune response<sup>26</sup>. Antigen-specific antibodies induced tumor cell lysis by the activation of complement-dependent cytotoxicity, and a large number of Th cells supported the production of IFN- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-17. This pro-inflammatory environment limited both myeloid suppressor cells and T regulatory cells<sup>26</sup>. Unfortunately, the DNA vaccination had a time-limited effect, and in the absence of booster vaccines, levels of both suppressor and regulatory cells increased again, reaching similar levels observed in untreated tumor-bearing mice. Moreover, IFN- $\gamma$  and TNF- $\alpha$  have been recently demonstrated to induce senescence in tumor cells<sup>27, 28</sup>.



Here we have shown that PDA cells obtained from a mouse model of autochthonous pancreatic cancer very weakly express MHC class I molecules as well as APM, which represents a big advantage for tumor escape from T cell-mediated immunity; loss or low expression of HLA class I and II is a general feature of PDA<sup>20-22</sup>. Of particular note, we observed that IFN- $\gamma$  treatment not only restored the expression of MHC class I, but also of most of the APM, suggesting a regulatory rather than a structural defect.

We demonstrated that ectopic expression of the MHC class II-transcriptional activator CIITA in the murine PDA cells rendered them more “visible” to the immune system so that they are subsequently rejected by 80 to 100% of syngeneic mice. Rejection is accompanied by the development of a long-lasting immune memory response that completely inhibited the growth of parental PDA cells.

Immunohistochemical analysis of the tumor area confirmed a great influx of T cells that contribute to the tumor rejection. *Ex vivo* evaluation of T cytotoxic activity revealed that both T cell populations are crucial for tumor cell lysis when T cells are derived from mice injected with sense CIITA-transfected cells, but not from mice injected with antisense CIITA-transfected cells. These data confirm the ability of CIITA-transfected cells to process full-length and endogenous antigens, and not only to present exogenous peptides which has already been demonstrated in an *in vitro* model for human PDA cells<sup>19,29</sup>. It is accepted that immune response efficacy for antigen-specific tumor rejection not only depends on correct antigen presentation by dendritic cells and activation of cytotoxic CD8 T cells, but also on the quality of CD4 T cell reactivity<sup>30,31</sup>. In this light, CIITA facilitated adequate antigen availability of highly tumorigenic PDA cells, which efficiently triggered tumor-specific CD4 Th cells that in turn activated CTL. Adequate antigen availability, in fact, has been proposed as a key player in triggering adaptive immune functions, which result in subversion from a pro-tumor to an anti-tumor microenvironment, tumor rejection and acquisition of an anti-tumor immune memory<sup>32</sup>. The importance of CD4 T cell priming in generating and maintaining efficient antitumor responses and tumor rejection after CIITA-tumor vaccination has been demonstrated by the fact that CD4 T cells alone are able to protect naive animals from tumor

growth in adoptive cell transfer assays, both in the case of sarcoma and colon tumors. This protection has been accompanied by the induction of strong cytolytic activity by CD8<sup>33</sup>. Overall, the results, as a whole, support the importance of antitumor-specific primed CD4 T cells in triggering naive antitumor CD8 T cell precursors to become fully differentiated functional CTLs, even if in the context of highly aggressive tumors. These considerations are, of course, important in understanding how chemo- and radio-therapy can be useful in helping the immune system to eradicate tumors, and in the design of novel vaccination strategies<sup>34</sup>.

The ability of PDA cells to re-express MHC class I, class II and APM when in the presence of cytokines such as IFN- $\gamma$  should also be recalled. This should encourage further efforts in designing novel curative approaches as, even in the presence of tumor cells that express low levels of antigen processing and presenting molecules, therapeutic approaches eliciting pro-inflammatory cytokines such as IFN- $\gamma$  may still be successful. Therefore, efficient vaccination or adoptive transfer strategies should be implemented for stimulating cytokine secretion by CD4 Th cells that support CTL differentiation, as discussed above, but also to edit tumor cells to act as “surrogate” antigen-presenting cells. PDA MHC class II expression induced by inflammatory processes in tumor tissues is also clinically relevant, as it significantly correlated with the infiltration of CD4 T cells, and with a better histological grade of PDA differentiation<sup>29</sup>. This can explain the significant amount of autoantibodies in cancer patients and in PDA patients. Indeed, by serological proteomics, we have identified a dozen of PDA-associated antigens that are recognized by patient autoantibodies<sup>35</sup>. The generation of a humoral response in PDA patients implies the coordinated activation of antigen-specific CD4 T cells, as recently demonstrated for ENO1 antigen<sup>25</sup>. Moreover, generation of T cell clones from PDA biopsies revealed, in fact, the presence of up to 20% of ENO1-specific CD4 T cells that infiltrate pancreatic cancer tissues. Of particular note, these CD4 T cells were able to exert cytolytic activity against tumor cells<sup>36</sup>.

Overall, this study strongly supports the key role of CD4 T cells in eliciting efficient integrated humoral and cellular anti-tumor responses in PDA. This translates as having a strong impact for the development of novel therapeutical strategies in clinical practice, particularly in pancreatic cancer.

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**Figure 1. MHC class I expression pattern of the murine PDA DT6606 and K8484 cell lines.** **A.** MHC class I surface expression H-2 K<sup>b</sup> (*black line*) and H-2 D<sup>b</sup> (*gray line*) was analyzed in basal conditions and after treatment with IFN- $\gamma$  (100 U/ml) for 48 h. Gray peaks represent cells stained with isotype-matched control Ig. A representative example of three independent experiments is shown. **B.** Transcription levels of H-2 class I heavy chains,  $\beta$ 2-microglobulin ( $\beta$ 2m), and several APM components, detected by real-time RT-PCR. Expression levels of the genes of interest were determined relative to levels of  $\beta$ -actin and *Gapdh* housekeeping genes. Data for TC-1, the MHC class I positive cell line, are set to 1. Values are depicted as mean  $\pm$  SD of three independent experiments performed in quadruplicate. A significance level of  $p \leq 0.001$  was found in all analyzed genes.

**Figure 2. CIITA-dependent MHC class II cell surface expression in PDA cells.** MHC class II profile of stable CIITA-transfected DT6606 and K8484 tumor cells was analyzed by flow cytometry. Isotype-matched control stained cells are represented with the light gray histograms while anti-I-A/E stained cells with the black histograms.

**Figure 3. CIITA-transfected tumor cell lines are rejected or drastically inhibited in their *in vivo* growth.**  $1 \times 10^5$  of sense-CIITA (black circles) or antisense (empty circles) K8484 (A)- or DT6606 (B)-transfected cells were injected subcutaneously (s.c.) in syngeneic mice (n=10 mice in two experiments). Tumor growth was monitored twice a week and results are expressed as mean of tumor diameter  $\pm$  SEM from two independent experiments. Sense-CIITA (black circles) or antisense-CIITA (empty circles) cell immunized mice were injected with  $1 \times 10^5$  of parental K8484 (C) or DT6606 (D) PDA cells 24 days after immunization. Tumor growth was monitored as before and results are expressed as mean of tumor diameter  $\pm$  SEM from two independent experiments.



**Figure 4. CTL responses from mice challenged with PDA-CIITA sense and antisense transfected cells.** Cytotoxic activity against DT6606 or K8484 parental cells was measured with a LDH-release assay. Cytotoxicity was measured at three different ratios of effector/target cells (E:T ratio). Blocking antibodies anti-MHC class II (I-A<sup>b</sup>) and/or anti-MHC class I (H-2K<sup>b</sup> /H-2D<sup>b</sup>) mAb were added to target cells before the cytotoxic assay. Different symbols represent the percentage of cytotoxicity mediated by effector T cells from CIITA-K8484 or -DT6606 cell immunized mice; the dotted line represents the percentage of cytotoxicity mediated by T cells from CIITA antisense-K8484 or -DT6606 cell immunized mice. Graph represents the mean  $\pm$  SEM of triplicate conditions in one of two independent experiments.

**Figure 5. CD4 and CD8 T cell recruitment analysis in CIITA sense- and antisense-transfected PDA by immunohistochemistry.** To evaluate infiltration of CD4 and CD8 T cells in tumor tissue from mice injected with sense CIITA K8484 (A, E) or DT6606 (B, F) transfected cells compared to those from mice injected with antisense CIITA K8484 (C, G) and DT6606 (D, H), paraffin-embedded tissues were stained with specific mAb. Magnification 40X.