Phosphorylated alpha-enolase induces autoantibodies in HLA-DR8 pancreatic cancer patients and triggers HLA-DR8 restricted T-cell activation.

(Article begins on next page)

30 September 2022

Phosphorylated Alpha-enolase induces autoantibodies in HLA-DR8 pancreatic cancer patients and triggers HLA-DR8 restricted T-cell activation

Michela Capello[1,3*], Cristiana Caorsi[2*], Pablo Josè Bogantes Hernandez[2], Ennia Dametto[2], Francesca Eleonora Bertinetto[2], Paola Magistroni[2], Sabina Rendine[4], Antonio Amoroso[2,4], Francesco Novelli[1, 2, 3]

[1]*Centre for Experimental Research and Medical Studies (CERMS) and [2]Immunogenetic and Transplant Biology Service, Azienda Ospedaliera Universitaria Città della Salute e della Scienza di Torino, Turin; [3]Department of Molecular Biotechnology and Health Sciences and [4]Department of Medical Sciences, Turin University, Turin, Italy.*

[*]M.C. and C.C. equally contributed to this work

Short title: Phosphorylated Alpha-enolase, pancreatic cancer and HLA-DR8.

Correspondence: Francesco Novelli, Centre for Experimental Research and Medical Studies (CERMS), Azienda Ospedaliera Universitaria Città della Salute e della Scienza di Torino, via Cherasco 15, 10126 Turin, Italy; Tel.: +390116336886 Fax: +390116336887 E-mail: franco.novelli@unito.it

Conflict of interest disclosure

The authors have declared no conflicting interests.

Abbreviations: Alpha-enolase (ENOA), Pancreatic ductal adenocarcinoma (PDAC), tumor-associated antigen (TAA), two-dimensional electrophoresis Western blot (2-DE WB), Fetal bovine serum (FBS), EBV-transformed lymphoblastoid B cell lines (EBV-B cells), [methyl-3H] thymidine (^{3}HTdR), Odds Ratio (OR).

Keywords: Pancreatic cancer, Alpha-enolase (ENOA), Autoantibodies, HLA-DR.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC), the fourth leading cause of cancer-related death in developed countries, is a lethal disease with an overall 5-year survival rate of <5% [1]. Due to the paucity of early symptoms, along with aggressive tumor behavior, less than 20% of patients present localized and resectable diseases at the time of diagnosis, and standard therapies only have a limited beneficial impact on advanced-stage patients [2]. Currently, attempts are being made to identify novel biomarkers to diagnose and predict PDAC outcome.

We have previously described Alpha-enolase (ENOA) as a tumor-associated antigen (TAA) in PDAC [3]. ENOA is a moonlighting protein that works both as a glycolytic enzyme and as a membrane plasminogen receptor, thus promoting tumor proliferation, spreading and metastasis [4, 5]. In particular, a fraction of PDAC patients specifically produce autoantibodies against two Ser^{419} phosphorylated isoforms of ENOA (ENOA1,2), which are over-expressed in PDAC [3, 4, 6, 7]. The detection of autoantibodies against ENOA1,2 positively complements the diagnostic performance of the commonly used serum marker CA19.9. Moreover, patients with autoantibodies against ENOA1,2 have a more favorable clinical course, with a longer progression-free and overall survival rate upon gemcitabine treatment [3].

A variety of post-translational modifications of naturally processed peptides displayed by MHC class I or II molecules have recently been described [8]. Among the post-translationally modified peptides identified to date, phosphopeptides are of particular interest, as deregulated phosphorylation is one of the major hallmarks of malignant transformation. T cell discrimination of phosphopeptides versus their unphosphorylated counterparts has also been observed, indicating that phosphorylation can influence peptide immunogenicity [9, 10, 11, 12]. Depending on the position within the peptide, phosphorylation tends to stabilize the complex, without greatly altering the peptide-binding mode.

4

The prognostic value of autoantibodies to ENOA1,2, together with the ability of ENOA to induce a specific CD4^{+} T cell response, led us to investigate whether the ability to produce autoantibodies against ENOA1,2, in PDAC patients, is associated with specific HLA-DRB1 alleles. Here, we demonstrate that expression of the HLA-DRB1*08 allele is greater in PDAC patients with autoantibodies to ENOA1,2 compared to healthy controls or patients lacking these antibodies. We found that a bioinformatically-predicted Ser[419]phosphorylated-ENOA peptide, which binds with high affinity to the HLA-DR8 molecule coded by HLA-DRB1*08:01 or *08:04 alleles, was able to activate *in vitro* proliferation of specific CD4^{+} T cell clones derived from a HLA-DRB1*08:01 donor.

2. Materials and Methods

2.1. Human samples. The study was approved by the Ethical Committee of Azienda Ospedaliera Universitaria Città della Salute e della Scienza di Torino, Turin and was in accordance with the 1964 Helsinki declaration. Serum samples and PBMCs were isolated from venous blood at the time of diagnosis, with the informed consent of the PDAC patients, and stored at −80°C until use. De-identified numeric specimen codes were used to protect the identity of the individuals. Diagnosis of PDAC was consistently confirmed by histological or cytological analysis. A total of 113 Italian PDAC patients, with clinical features described in **Supplementary Table 1**, were screened for reactivity against ENOA isoforms by two-dimensional electrophoresis Western blot (2-DE WB), as described previously [3]. Briefly, cells (10^7) from the CF-PAC-1 cell line (ECACC ref. No. 91112501) were solubilized, subjected to 2DE and electro-transferred onto a nitrocellulose membrane (GE Healthcare Bio-Sciences, Uppsala, Sweden). Sera were individually tested on 2DE maps at a working dilution of 0.1 mg/ml IgG for 4 h, followed by incubation with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (90 minutes, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a secondary antibody. Immunodetection was accomplished by ECL PLUS (Enhanced Chemiluminescence, GE Healthcare Bio-Sciences) and the

5

resulting chemifluorescent signals were scanned with "ProXPRESS 2D" (PerkinElmer). The volume of ENOA1,2 spots recognized by autoantibodies was calculated after background subtraction using "ProFinder 2D" (PerkinElmer) software. Out of 113 patients, 104 were evaluable for ENOA1,2 reactivity and 36 of them (35%) were positive for the presence of ENOA1,2 autoantibodies. Their genomic DNA was obtained from PBMCs, using a NucleoSpin® Blood kit (Macherey-Nagel Gmbh & Co. KG) on an automatic workstation Biomeck 2000 (Beckman-Coulter, Inc., Milano, Italy). The control group was composed of about 261,000 Italian healthy bone marrow donors (listed in the Italian Bone Marrow Donor Registry-IBMDR), previously typed with serological or molecular techniques [13]. To generate T cell clones and autologous EBV-B cell lines, a HLA-DRB1*08 healthy donor was selected from the IBMDR and enrolled after written informed consent.

2.2. HLA typing. Low resolution HLA-DRB1 typing of PDAC patients was performed using a LABType® SSO kit (RSSO2B1, One Lambda-Thermo Fisher Scientific, Inc., CA, USA), based on the SSO-reverse principle, on a Luminex® 100™ instrument. High resolution of HLA-DRB1*08 positive samples was obtained using the Sequence Based Typing (SBT) method with an Allele SEQR HLA-DRB1 Core kit (Abbott Laboratories, Abbott Park, Illinois, USA) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. Prediction of possible HLA-DRB1 ligands of the ENOA C-terminus. Possible HLA-DRB1 ligands of the C-terminus region of ENOA[410-432] were predicted using the bioinformatics tool IEDB (http://tools.immuneepitope.org/mhcii/) [14]. Based on the availability of predictors and previously observed predictive performance, IEDB uses the best possible method for predicting binding of a given MHC molecule from the currently implemented MHC class II binding prediction methods, namely: Consensus method, Combinatorial library, NN-align (netMHCII-2.2), SMM-align (netMHCII-1.1), Sturniolo, and NetMHCIIpan. For each predicted binding peptide, a median percentile rank of each applied method was generated by comparing the score of the peptide against the scores of five million random 15mers selected from the SWISSPROT database. A numerically

low percentile rank indicates a high affinity. Predictions were further confirmed by means of other publicly available methods of prediction (NetMHCII, http://www.cbs.dtu.dk/services/NetMHCII/; HLAPred, http://www.imtech.res.in/raghava/hlapred/; ProPred http://www.imtech.res.in/raghava/propred/; and SVMHC, http://abi.inf.uni-tuebingen.de/Services/SVMHC).

2.4. Generation of Ser[419]P-ENOA-specific CD4^{+} T cells. Peptide–specific CD4^{+} T cells were obtained after two rounds of *in vitro* stimulation of PBMCs from a HLA-DRB1*08:01 healthy donor. Briefly, PBMCs were cultured in 24-well plates in RPMI 1640 medium plus 10% heat-inactivated certified FBS (both from Invitrogen Life Technologies, Italy) in the presence of the Ser[419]P-ENOA synthetic peptide ([412]RIEEELGSpKAKFAGRNFRN[430]) (10 µg/ml; China Peptides Co., China). Recombinant IL-2 (10U/ml) (Proleukin, Novartis Farma, Italy) was added on day 5, and replenished every 3-4 days. T cells were re-stimulated for two rounds with irradiated autologous PBMCs pulsed with Ser[419]P-ENOA, and T cells were then cloned for limited dilution, as previously described [15]. Long term CD4^{+} T cell clones were re-stimulated every 10-14 days with irradiated feeder cells.

2.5. Generation of HLA-DRB1*08:01 EBV-B cell lines. To obtain EBV-transformed lymphoblastoid B cell lines (EBV-B cells), PBMCs from a HLA-DRB1*08:01 healthy donor were incubated overnight with the supernatant from the EBV-producing marmoset cell line B95.8 (kindly provided by Dr. Ada Funaro, University of Turin), and subsequently expanded in complete RPMI 1640 medium plus 15% heat-inactivated certified FBS in the presence of irradiated allogeneic feeder cells.

2.6. T cell proliferation assay. To assess specific peptide recognition, 2×10^4 cells per well were cultured in flat bottomed 96-well plates in RPMI 1640 medium plus 4% heat-inactivated certified FBS, 10:1 with autologous irradiated EBV-B cells that had been pulsed overnight with 20 µg/ml Ser[419]P-ENOA or the corresponding unphosphorylated form. After 2 days, 1 µCi of [methyl-3H] thymidine ([3]HTdR, 25 Ci/mmol) (PerkinElmer Life Sciences, Belgium) was added to each well, and

incubated for 18 h. Cells were collected using a CELL Harvester (PerkinElmer Life Sciences) in UNIfilter plates, and ^{3}HTdR uptake was quantified (TopCount Microplates Scintillation Counter; PerkinElmer Life Sciences). All tests were performed in quadruplicate. In some assays growing amounts of peptides were used for pulsing EBV-B cells (2, 20 and 200 μg/ml, respectively).

2.7. Statistical analysis. The allelic frequencies were derived from the direct count of genes. The Pearson's chi-squared test or Fisher's exact test were used to compare observed allele frequencies, as appropriate. Differences were considered statistically significant when p<0.05. Odds ratios (OR) were used to estimate the measure of relative risk (CI 95%). The Student's t-test, calculated using Graph-Pad Software Inc. (Version 5, San Diego, CA, USA), was used to evaluate the differences in proliferation of CD4$^+$ T cell clones.

3. Results

3.1. HLA-DRB1 allelic frequencies in PDAC patients and production of autoantibodies to ENO1,2.

The presence of circulating autoantibodies to the PDAC-associated ENOA1,2 isoforms was assessed by 2-DE WB in 113 PDAC patient sera, using CF-PAC-1 cell lysates as source of antigen. The HLA-DRB1 allelic frequencies of the same patients were in parallel analyzed with low resolution methods. In our cohort, out of 113 patients, 104 were evaluable for ENOA1,2 reactivity and 36 of them (35%) were positive, while 68 patients were negative for the presence of ENOA1,2 autoantibodies (Supplementary Table 1). HLA-DRB1*08 allelic frequency was significantly higher (8%, 6/72 alleles) in patients with autoantibodies to ENOA1,2 (ENOA1,2$^+$) than in healthy controls (3%, 15,648/521,602 alleles; p=0.0112; OR= 2.8141 [1.2199-6.4918]). Moreover HLA-DRB1*08 allelic frequency was higher in ENOA1,2$^+$ patients respect those lacking these autoantibodies (ENOA1,2$^-$) (2%, 3/136 alleles) (**Figure 1**). Even if borderline significant (p=0.067) due to the small sample size, this trend prompt us to hypothesize a correlation between ENOA1,2 autoantibody production and a better presentation of phosphorylated-Ser^{419}ENOA by the HLA-DR8 molecule.

3.2. Prediction of possible HLA ligands of the ENOA C-terminus.

High resolution typing of HLA-DRB1*08 PDAC patients (n=9) showed that 89% of patients were HLA-DRB1*08:01 or *08:04 (n=8/9; relative frequencies: 67% and 22%, respectively), (**Supplementary Table 2**), in line with the results observed in healthy controls from the Caucasian population (71% and 12%, respectively [16]). Based on this observation, we employed bioinformatics tool IEDB (http://tools.immuneepitope.org/mhcii/) to predict possible HLA-DRB1 ligands of the C-terminus region of ENOA$^{410-432}$, which contains Ser^{419} from the HLA-DRB1*08:01 or *08:04 alleles (**Table 1**). Notably, we confirmed that DRB1*08:01 and *08:04, along with

DRB1*13:01, were the alleles that had the highest affinity for the C-terminus of ENOA[411-431] (**Table 1**). The predicted binding core in this portion of ENOA is the 9mer [417]LGSKAKFAG[425], where the primary anchor residue is Leu[417] (P1), and the phosphorylable residue Ser[419] is in the position P3. It has been demonstrated that the phosphomoiety stabilizes the MHC class II-TCR complex, without altering the peptide-binding mode, when it is not present in a primary (P1, P6) or secondary anchor (P4, P7, P9)[17]. The 9mer [417]LGSKAKFAG[425] was also predicted by other publicly available methods (NetMHCII, HLAPred, ProPred and SVMHC), together with additional ENOA peptides able to bind to the HLA-DR8 molecule (**Supplementary Figure 1**).

3.3. Specific CD4^{+} T cell recognition of the Ser[419]P-ENOA peptide.

Since the four N-terminal residues in positions P4 to P1 of the binding core of a tumor-associated MHC class II-restricted phosphopeptide include structural determinants required for TCR recognition [17], in order to perform functional studies, we synthesized a Ser[419]P-ENOA peptide ([412]RIEEELGSpKAKFAGRNFRN[430]), along with the corresponding unphosphorylated ENOA peptide ([412]RIEEELGSKAKFAGRNFRN[430]), which was 10 amino acids longer than the predicted 9-mer HLA-DRB1*08 binding peptide ([417]LGSKAKFAG[425]).

PBMCs from a HLA-DRB1*08:01 healthy donor were repeatedly stimulated *in vitro* with the Ser[419]P-ENOA peptide. In long-term CD4^{+} T cell cultures, 21 CD4^{+} T cell clones were selected, which were able to proliferate in response to the autologous EBV-B cells pulsed with Ser[419]P-ENOA (data not shown). Five of these CD4^{+} T cell clones showed a significant proliferative response to the Ser[419]P-ENOA, which was higher than that induced by the unphosphorylated peptide (**Figure 2A**). Moreover, using increasing concentrations of the Ser[419]P-ENOA peptide for pulsing of autologous EBV-B cells, a dose-dependent proliferation of CD4^{+} T cell clones was observed (**Figure 2B**). These data indicate that CD4^{+} T cells specifically recognize Ser[419]P-ENOA, demonstrating that the phosphorylated serine residue is a critical determinant of recognition.

4. Discussion

In this study we demonstrate, for the first time, an association between the presence of the HLA-DRB1*08 allele and the production of autoantibodies against a phosphorylated epitope of a specific TAA, namely ENOA. We have shown that HLA-DRB1*08 allele is more frequent in PDAC patients with autoantibodies against Ser^{419}P-ENOA than healthy controls or patients lacking these autoantibodies. Notably, an ENOA-derived peptide containing Ser^{419} was bioinformatically-predicted to bind with high affinity to the HLA-DR8 molecule coded by HLA-DRB1*08:01 or *08:04 alleles. The Ser^{419}P-ENOA peptide was able to induce proliferation of HLA-DR8-restricted CD4$^+$ T cell clones due to the phosphate moiety of the peptide, presented in the context of HLA-DRB1*08, to a greater extent than the corresponding unphosphorylated peptide.

We have previously reported that two Ser^{419} phosphorylated isoforms of the glycolytic enzyme ENOA (ENOA1,2) elicit the production of autoantibodies in a subset of PDAC patients, who show a more favorable clinical outcome [3, 6, 7, 18]. Several TAAs induce the production of IgG autoantibodies in cancer patients, by means of an integrated immune response triggered by CD4$^+$ T cells, CD8$^+$ T cells and B cells. TAAs released by tumor cells are captured from APCs, processed and presented by either MHC class I or class II molecules for priming and activation of CD8$^+$ and CD4$^+$ T cells, respectively. Activated CD4$^+$ T cells, through the secretion of appropriate cytokines, trigger B cells to produce IgG against the same TAA, and leads to differentiation of CD8$^+$ T cells into TAA-specific CTL [19, 20]. ENOA in PDAC can elicit a coordinated CD4$^+$ and CD8$^+$ T cell response both *in vitro* and *in vivo* and, in PDAC patients, the production of anti-ENOA IgG correlates with the ability of T cells to be activated in response to ENOA [21]. *Ex vivo* analysis of pancreatic cancer-infiltrating T lymphocytes has revealed that ENOA Th1/Th17 effector cells and T reg cells tend to accumulate in tumor tissue [15].

The prognostic value of Ser^{419}P-ENOA-autoantibodies, together with the ability of ENOA to induce a specific CD4$^+$ T cell response, prompted us to investigate whether, in PDAC patients, the ability

to produce autoantibodies against Ser^{419}P-ENOA is associated with the presence of specific HLA-DRB1 alleles. Here we show that the allelic frequency of HLA-DR8, coded by the HLA-DRB1*08:01 or *08:04 alleles, was significantly higher in PDAC patients with ENOA1,2 autoantibodies than in patients without autoantibodies or in healthy controls, suggesting a preferential presentation of Ser^{419}P-ENOA peptides by HLA-DR8.

The ENOA sequence shows several bioinformatically-predicted HLA-DR8 binding peptides, and the ability of ENOA-derived peptides to bind HLA-DR8 has also been previously reported in primary biliary cirrhosis and in oral squamous cell carcinoma [22, 23, 24, 25]. Interestingly, the C-terminus region of ENOA that contains Ser^{419} was predicted to bind with high affinity to the HLA-DR8 molecule, with the residue Ser^{419} in position P3 of the HLA-DR8 binding peptide. As the phosphomoiety stabilizes the MHC class II-TCR complex, without altering the peptide-binding mode when it is not present in a primary or secondary anchor [17], the same peptide is likely to be able to bind HLA-DR8 when the phosphorylation event occurs. This Ser^{419} phosphorylated peptide was synthesized according to the molecular basis for phosphopeptide MHC class II presentation, recently resolved by crystallographic analysis, and was able to activate specific $CD4^+$ T cell clones derived from a HLA-DRB1*08:01 donor. Synthetic peptides were chosen as Ser^{419}-ENOA phosphorylation seems to be pancreatic specific [3, 18], suggesting that ENOA would not be properly phosphorylated when expressed into EBV-transformed cells. As reported in previous studies in which T cells could discriminate phosphopeptides from their unphosphorylated counterparts [9, 10, 11, 12], the HLA-DR8-restricted $CD4^+$ T cell clones were specific for the phosphate moiety of the ENOA-derived peptide presented in the context of HLA-DR8. The crystal structure of phosphopeptide–MHC complexes shows, in fact, that the phosphomoiety is exposed to the TCR, suggesting a direct interaction between the phosphorylated residue and the TCR [8, 17, 26].

The identification of a CD4$^+$ T cell clone able to proliferate and produce IFN-γ after stimulation with ENOA-derived phosphopeptide strongly supports the hypothesis that the preferential production of IgG antibodies to Ser^{419}P-ENOA in HLA-DR8 patients is critically controlled by the activation of CD4$^+$ helper T cells that recognize the phosphopeptide in the context of HLA-DR8. It is likely that this ENOA-derived phosphopeptide is displayed by the HLA-DRB1*08 allele on the surface of B cells or other APCs and thus activates specific CD4$^+$ T helper cells, which in turn help B cells to produce antibodies against this tumor-associated ENOA-derived phosphopeptide. Although the frequency of the HLA-DRB1*08 allele is relatively low in Caucasian populations, its presence could be considered as a genetic factor that influences the production of autoantibodies against ENOA1,2.

In conclusion, our results suggest that Ser^{-419}P-ENOA peptide–MHC complexes can trigger T-cell signaling and induce an anti-tumor immune response, thus portraying ENOA not only as a diagnostic tool, but also as a promising candidate for PDAC immunotherapy. DNA-based vaccination against human ENOA is able to significantly prolong the median life expectancy of mice that spontaneously develop PDAC, by inducing both a humoral and cellular response [27]. Based on the findings described in this study, together with the prognostic value of Ser^{419}P-ENOA-autoantibodies, it can be speculated that the presence of HLA-DRB8 in PDAC patients can be an indicator of a better response to chemotherapy, as well as a potential marker for selecting candidates for DNA-based ENOA vaccination.

References

[1]   Jemal A, Siegel R, Xu J, Ward E. Cancer statistics. CA Cancer J Clin. 2010;60:277-300.

[2]   Hidalgo M. Pancreatic cancer. N Engl J Med. 2010;362:1605-17.

[3]   Tomaino B, Cappello P, Capello M, et al. Circulating autoantibodies to phosphorylated alpha-enolase are a hallmark of pancreatic cancer. J Proteome Res. 2011;10:105-12.

[4]   Capello M, Ferri-Borgogno S, Cappello P, Novelli F. alpha-Enolase: a promising therapeutic and diagnostic tumor target. FEBS J. 2011;278:1064-74.

[5]   Ceruti P, Principe M, Capello M, Cappello P, Novelli F. Three are better than one: plasminogen receptors as cancer theranostic targets. Exp Hematol Oncol. 2013;2:12.

[6]   Chiriaco MS, Primiceri E, Monteduro AG, et al. Towards pancreatic cancer diagnosis using EIS biochips. Lab Chip. 2013;13:730-4.

[7]   Bianco M, Aloisi A, Arima V, et al. Quartz crystal microbalance with dissipation (QCM-D) as tool to exploit antigen-antibody interactions in pancreatic ductal adenocarcinomadetection. Biosens Bioelectron. 2013;42:646-52.

[8]   Petersen J, Purcell AW, Rossjohn J. Post-translationally modified T cell epitopes: immune recognition and immunotherapy. J Mol Med (Berl). 2009;87:1045-51.

[9]   Zarling AL, Ficarro SB, White FM, Shabanowitz J, Hunt DF, Engelhard VH. Phosphorylated peptides are naturally processed and presented by major histocompatibility complex class I molecules in vivo. J Exp Med. 2000;192:1755-62.

[10]   Zarling AL, Polefrone JM, Evans AM, et al. Identification of class I MHC-associated phosphopeptides as targets for cancer immunotherapy. Proc Natl Acad Sci U S A. 2006;103:14889-94.

[11]   Depontieu FR, Qian J, Zarling AL, et al. Identification of tumor-associated, MHC class II-restricted phosphopeptides as targets for immunotherapy. Proc Natl Acad Sci U S A. 2009;106:12073-8.

[12]   Meyer VS, Drews O, Gunder M, Hennenlotter J, Rammensee HG, Stevanovic S. Identification of natural MHC class II presented phosphopeptides and tumor-derived MHC class I phospholigands. J Proteome Res. 2009;8:3666-74.

[13]   Amoroso A, Ferrero, N. M., Rendine, S. Sacchi, N. Le caratteristiche HLA della popolazione italiana: analisi di 370.000 volontari iscritti all'IBMDR. Analysis 2010;1/2:23-104.

[14]   Bui HH, Sidney J, Peters B, et al. Automated generation and evaluation of specific MHC binding predictive tools: ARB matrix applications. Immunogenetics. 2005;57:304-14.

[15]   Amedei A, Niccolai E, Benagiano M, et al. Ex vivo analysis of pancreatic cancer-infiltrating T lymphocytes reveals that ENO-specific Tregs accumulate in tumor tissue and inhibit Th1/Th17 effector cell functions. Cancer Immunol Immunother. 2013;62:1249-60.

[16]   Rendine S, Ferrero NM, Sacchi N, Costa C, Pollichieni S, Amoroso A. Estimation of human leukocyte antigen class I and class II high-resolution allele and haplotype frequencies in the Italian population and comparison with other European populations. Hum Immunol. 2012;73:399-404.

[17]   Li Y, Depontieu FR, Sidney J, et al. Structural basis for the presentation of tumor-associated MHC class II-restricted phosphopeptides to CD4+ T cells. J Mol Biol. 2010;399:596-603.

[18]   Zhou W, Capello M, Fredolini C, et al. Mass spectrometry analysis of the post-translational modifications of alpha-enolase from pancreatic ductal adenocarcinoma cells. J Proteome Res. 2010;9:2929-36.

[19]   Sahin U, Tureci O, Pfreundschuh M. Serological identification of human tumor antigens. Curr Opin Immunol. 1997;9:709-16.

[20]   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;421:852-6.

[21]   Cappello P, Tomaino B, Chiarle R, et al. An integrated humoral and cellular response is elicited in pancreatic cancer by alpha-enolase, a novel pancreatic ductal adenocarcinoma-associated antigen. Int J Cancer. 2009;125:639-48.

[22]   Chicz RM, Urban RG, Gorga JC, Vignali DA, Lane WS, Strominger JL. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. J Exp Med. 1993;178:27-47.

[23]   Akisawa N, Maeda T, Iwasaki S, Onishi S. Identification of an autoantibody against alpha-enolase in primary biliary cirrhosis. J Hepatol. 1997;26:845-51.

[24]   Sato N, Nabeta Y, Kondo H, et al. Human CD8 and CD4 T cell epitopes of epithelial cancer antigens. Cancer Chemother Pharmacol. 2000;46 Suppl:S86-90.

[25]   Kondo H, Sahara H, Miyazaki A, et al. Natural antigenic peptides from squamous cell carcinoma recognized by autologous HLA-DR8-restricted CD4+ T cells. Jpn J Cancer Res. 2002;93:917-24.

[26]   Mohammed F, Cobbold M, Zarling AL, et al. Phosphorylation-dependent interaction between antigenic peptides and MHC class I: a molecular basis for the presentation of transformed self. Nat Immunol. 2008;9:1236-43.

[27]   Cappello P, Rolla S, Chiarle R, et al. Vaccination with ENO1 DNA prolongs survival of genetically engineered mice with pancreatic cancer. Gastroenterology. 2013;144:1098-106.

Figure legends

Figure 1. HLA-DR allelic frequencies in PDAC patients based on ENOA1,2-reactivity, and healthy controls. Low resolution HLA-DR typing was performed in PDAC patients, previously classified according to ENOA1,2 reactivity (n=104; described in **Supplementary Table 1**), and healthy controls (n=260801). HLA-DR8 was significantly more frequent in PDAC patients with autoantibodies to ENOA1,2 than in healthy controls (8% vs. 3%, p=0.0112, allelic frequency) and is higher than in PDAC patients lacking these autoantibodies (8% vs. 2%, allelic frequency).

Figure 2. Specific CD4$^+$ T cell recognition of the Ser^{419}P-ENOA peptide. A) Proliferation of specific CD4$^+$ T cell clones against the autologous EBV-B cells that had been pulsed with Ser^{419}P-ENOA or the corresponding unphosphorylated control peptide (Ser^{419}ENOA). Symbols on the right show the p value relative to the comparison of Ser^{419}P-ENOA vs Ser^{419}ENOA for each T cell clone. **B)** Proliferation of specific CD4$^+$ T cell clones against the autologous EBV-B cells pulsed with growing concentrations of Ser^{419}P-ENOA (black symbols) or Ser^{419}ENOA (white symbols) of two CD4$^+$ T cell clones from panel A). The p values are: 0.002 at 2 ng/ml, 0.0213 and <0.0001 at 20 ng/ml, 0.0017 and 0.0003 at 200 ng/ml, respectively. The Stimulation Index (SI) was calculated as the ratio between the mean values of cpm (counts per minute) obtained from stimulated cultures, and those obtained in the presence of EBV-B cells in medium alone. Background cpm values from CD4$^+$ T cell clones in medium alone were always subtracted.

Vitae

Michela Capello After earning a Master's degree in Molecular Biotechnology in 2006, Michela obtained her PhD in Molecular Medicine in 2011, from the University of Turin, Italy, where she investigated the identification and functional characterization of tumor antigens in pancreatic ductal adenocarcinoma. Michela is currently a Post-Doctoral fellow at the Department of Clinical Cancer Prevention of the University of Texas, MD Anderson Cancer Center, Houston, TX, where she is conducting a Proteomics-based biomarker discovery in pancreatic cancer.

Cristiana Caorsi After graduating in Biological Sciences in 2000, Cristiana obtained a PhD in Immunology and Cellular Biology in 2004, from the University of Turin, Italy, where she studied the ability of chemokines and natural molecules to improve dendritic cell antigen presentation to trigger T cell activation. She has also investigated new immunotherapeutic strategies for cancer treatment. In 2006, Cristiana moved to the Karolinska Cancer Centrum in Stockholm, Sweden. Since 2010, she has collaborated with the Immunogenetic and Transplant Biology Service *Città della Salute e della Scienza* Hospital of Turin by performing studies on the human leukocyte antigen (HLA) and transplant tolerance.

Pablo Josè Bogantes Hernandez graduated in Medicine and specialized in Pediatrics at the University of Alajuela, Costa Rica. Upon successfully gaining an ERACOL fellowship, Pablo spent 1 year working at the Immunogenetic and Transplant Biology Service, *Città della Salute e della Scienza* Hospital of Turin, performing HLA molecular typing.

Ennia Dametto graduated in Biological Sciences from University of Turin, Italy, followed by specialization in Clinical Pathology at the University of Turin. Since 1999, Ennia has been working at the Immunogenetic and Transplant Biology Service, *Città della Salute e della Scienza* Hospital of Turin, where has gained expertise in HLA molecular typing, immunogenetic, hematopoietic stem cell (CSE) and solid organ transplantation.

Francesca Eleonora Bertinetto After graduation in Biological Sciences, Francesca specialized in Clinical Pathology at the University of Turin. Currently, she has a post-graduate position at the Immunogenetic and Transplant Biology Service, *Città della*

Salute e della Scienza Hospital of Turin. Her research experience is in HLA and transplantation, typing activity for Italian Bone Marrow and Cord Blood Registry of Turin, HLA-associated diseases and genetic polymorphisms.

Paola Magistroni After graduating in Physics, Paola is currently employed as a data analyst at the Immunogenetic and Transplant Biology Service, *Città della Salute e della Scienza* Hospital of Turin. For more than 15 years she has been primarily involved in software development and data management at the Regional Registry of Transplantation.

Sabina Rendine After graduating in Computer Science from the University of Turin, Sabina has been carrying out research activity since 1984 at the Medical Sciences Dept. of Turin University. Sabina taught Medical Statistics at the Faculty of Medicine and Surgery of the University of Turin until 1996. Her research interests include the study of genetic variability and evolution of human populations, analysis of genetic diversity within the Italian population, with particular focus on the HLA system. She is also involved in management and statistical analyses of the Turin Cord Blood Bank and of the regional Bone Marrow Donor Registries.

Antonio Amoroso graduated in Medicine and specialized in Immunohematology. In 1988 he became Assistant Professor in the Transplantation Immunology Service, Molinette Hospital of Turin, Institute of Medical Genetics, where he became Associate Professor in 1992. In 1996 he moved to Trieste to head the Medical Genetics Dept. at the Faculty of Medicine, Trieste University. Since 1999 he has been Full Professor of Medical Genetics, in 2004 he became the Director of the Transplantation Immunology Dept. of the Molinette Hospital of Turin. In addition to teaching courses in the Faculty of Medicine, he has authored about 200 publications in high IF scientific journals.

Francesco Novelli is Associate Professor of Immunology at the Medical School of Turin University. Since 2001, Francesco has been co-director of the Tumor Immunology Laboratory, *Città della Salute e della Scienza* Hospital of Turin. He has acquired significant expertise in identification and characterization of novel tumor-associated antigens (TAAs) by using a proteomic approach, and in developing preclinical models of pancreatic cancer to design novel immunotherapeutic strategies, particularly based on DNA vaccination. He has also studied the humoral response of pancreatic cancer patients and genetically-modified mice developing spontaneous

cancer in order to identify specific autoantibodies to TAAs for exploitation in early cancer diagnosis.