PROJECT FINAL PUBLIC SUMMARY

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Project acronym: TUMADOR

Project title: Development of very promising humanized therapeutic mAbs efficiently neutralizing a novel immunosuppressive pathway involved in a wide range of cancers

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1. Final publishable summary report

1.1 Executive summary

Based on the broad immuno-suppression and strong tumor promotion (proliferation and dissemination) exerted by adenosine, therapeutic strategies aimed at targeting adenosine pathway are highly promising. However, given involvement of the four known specific adenosine receptors, the use of mAbs neutralizing the enzymatic function of CD73, the sole 5' ecto-nucleotidase expressed at surface membrane, thus preventing dephosphorylation of AMP into adenosine, represents an attractive, potent and safe strategy for the development of innovative immunotherapy.

The TumAdoR project aims at paving the way to clinical trials of potent neutralizing anti-CD73 mAb candidates in cancer patients. It should validate a novel cancer immunotherapy approach applicable to a wide range of cancers preventing dissemination and relapse through restoration of anti-tumour immune memory.

The program has progressed very well with the development and selection of the lead candidate for a neutralizing anti huCD73 mAb. The final lead candidate anti-huCD73 Ab has been humanized, and produced in large batches for safety evaluation. The lead candidate has been further characterized and compared with the Abs from the competitors, and back-up anti-huCD73 Abs have been generated.

Many novel data on huCD73 biology on $CD4^+$ T cells and A2A functionality on $CD8^+$ T cells and NK cells have been generated, increasing the relevance of CD73 therapeutic targeting. Current data also indicate lack of safety issues.

Furthermore, standardized tools for IHC (automated protocol and quantification grid) have been developed for the analysis of CD73 target expression on large cohorts of human solid tumors. Several partners of the project have generated knowledge on CD73 distribution in human selected tumors and its impact on tumor progression and patient survival allowing a comprehensive huCD73 expression that will be critical for the future orientation of the clinical development. A new mAb has been selected, for use in future IHC companion diagnostic for anti-huCD73 treatment.

A major investment has been made by *the partners* to develop mouse models for *in vivo* evaluation of anti-CD73 mAbs, despites technical difficulties the consortium select relevant models to assess CD73 biology in vivo and the impact of the drugs on immune parameters. Finally, tools for the immune-monitoring of the future clinical trial with the drug candidate are now all in place.

The results obtained in the course of TumAdoR have been presented at several scientific events (31 dissemination activities, 1 PhD, 3 publications, 8 presentations, 14 posters, others 5).

1.2 Summary description of project context and objectives

Cancer is a clinical and societal burden that justify important Research and Development effort to better understand the biology of these disease and their spread, prerequisite for the identification of new therapeutic pathway and potent clinical tools. Among them monoclonal antibodies (mAbs) for targeted

cancer therapies has generated in the last decade a lot of enthusiasm and hope for patients and physicians in the frame of more efficient, safer, personalized medicine. The mAbs oncology market is constantly growing, reaching nearly 20 billion Euros in 2012. One of the aims of the TumAdoR project is to boost the EU competitiveness of health-related industries and businesses, as well as address global health issues.

The major challenge for success that has to be addressed in cancer immunotherapy is that tumors fool the host immune system, favoring tumor spread and shortening patient survival. Some tumor-driven immunomodulating mechanisms have been identified: secretion of immunosuppressive cytokines, recruitment of suppressive immune cells, local amino-acid starvation, altered activation of immune receptors and engagement of inhibitory receptors. In addition to being involved in the natural progression of cancer, this immunosuppressive environment affects the efficacy of anticancer treatments. At the beginning of TumAdoR project in 2013, few targets contributing to tumor immune-suppression have been clinically evaluated using neutralizing mAbs and the successes in clinical trials of anti-PD1 and PD-L1 mAbs opened the door to a highly promising novel class of therapeutics targeting tumor-driven immunosuppressive pathways. However, as severe autoimmune syndromes related to disruption of immune tolerance have been reported after treatment with anti-CTLA-4 mAbs, development of strategies targeting other tumor-driven immunosuppressive pathways with increased efficacy and lower toxicity was urgently needed.

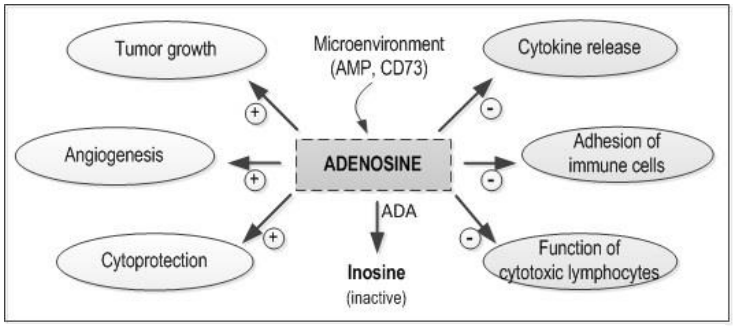

Figure 1: Impact of Ado accumulation on tumor (White: direct effect on tumor cells or angiogenesis, Grey: indirect effect through the immune system).

Extracellular Ado has multiple activities that contribute to tumor progression (**Figure 1**). Ado has direct effects on tumor cells contributing to their proliferation and favoring tumor cell motility and metastases (for review Allard 2012). Furthermore Ado has direct effect on endothelial cells and increase angiogenesis and protects cancer cells from hypoxia (Ahmad 2009). In addition to these direct tumor promoting activities, Ado has critical immunomodulatory properties, suppresses both innate and adaptive immune responses and inhibits pro-inflammatory cytokine production. Finally, Ado inhibits immune cell recruitement by inhibiting expression of adhesion molecules on endothelial cells and T cells.

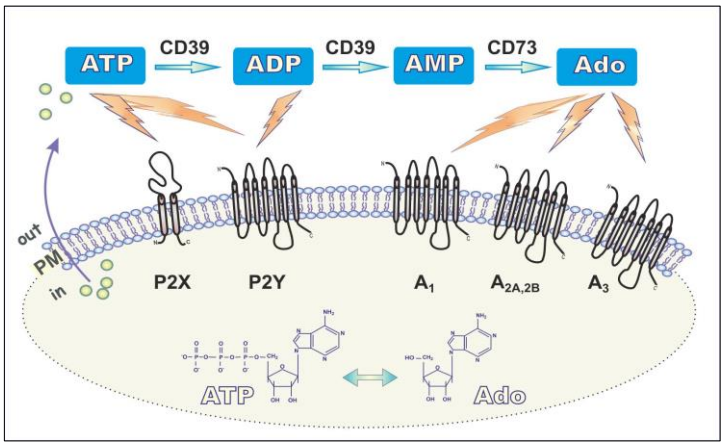

Figure 2: Extracellular ATP metabolism

The conversion of ATP into Ado is tightly regulated by cell membrane ectoenzymes of the NTPDase family and tissue non-specific alkaline phosphatases (**Figure 2**). The conversion of ATP into AMP is predominantly catalyzed by CD39. CD73, a glycosylphosphatidylinositol (GPI)-anchored nucleotidase present in cell membrane lipid rafts, active as a disulfide-linked (for review Yegutkin 2008). Importantly, whereas the activity of CD39 could be reverted by the action of NDP kinase and adenylate kinase,

CD73 activity is virtually irreversible thus representing a crucial checkpoint in conversion of pro-inflammatory ATP into immunosuppressive Ado. Indeed, ATP is a danger signal released by damaged and dying cells that primes immune responses through the ligation of P2X and P2Y purino-receptors. By contrast, through the activation of G-protein-coupled seven transmembrane spanning receptors (A2A, A2B), Ado suppresses the immune responses of not only $CD4^+$ and $CD8^+$ T cells, but also of the antigen presenting cells, NK and NKT cells (for review Stagg 2010).

Thus, based on the broad immuno-suppression and strong tumor promotion (proliferation and dissemination) exerted by adenosine (Ado), therapeutic strategies aimed at inhibiting adenosine appeared highly promising. For one research team at the Centre Anticancéreux Léon Bérard (CLB, coordinator of TumAdoR project) the use of mAbs neutralizing the enzymatic function of CD73 receptor expressed at surface membrane, thus preventing the generation of Ado, represented an attractive strategy for the development of innovative immunotherapy. In addition, CD73 neutralization appears to reduce tumorigenesis and metastasis. Combining the expertise of 6 top-level scientific partners in Europe and the US, the TumAdoR project aims at paving the way to clinical trials of potent neutralizing anti-huCD73 mAb candidates in cancer patients. Neutralizing CD73 activity is expected to be efficient in a wide range of cancers, and to have limited adverse side effects.

TumAdoR project should in the coming years validate a novel cancer immunotherapy approach. This novel approach will enable the development of specific anti-tumor immune response inhibiting cancer progression and dissemination and preventing relapse through restoration of anti-tumor immune memory.

Specifically, the objectives of the TumAdoR program were to 1- generate a comprehensive knowledge of CD73 distribution in tissues and major type of tumors, 2- understand CD73 biology as an immune checkpoint in human, 3- develop therapeutic antibodies neutralizing CD73 enzymatic activity, 4- set up new preclinical models for relevant in vivo therapeutic evaluation of anti-CD73 therapeutic candidate, 5- develop specific assays for toxicity evaluation of anti-CD73 therapeutic candidate, 6- set new Immuno-monitoring tools to monitor anti-CD73 mAb clinical trial.

1.3 Main S&T results/foregrounds

Progress in Target and associated biomarkers expression, and clinical correlation on survival/relapse.

Information on CD73 prevalence in human tumor types and its impact on tumor progression and patient survival was limited, so the consortium undertook the development of specific tools to analyze CD73 expression and markers associated with CD73 biology on well clinically annotated cohorts of selected tumors types.

A commercial antibody was selected that provided specific and unambiguous staining (rabbit mAb D7F9A, Cell Signalling Technology). In addition, a short-list of biomarkers associated with CD73 and its biological function has been generated through bioinformatics analysis. The evaluation, harmonization and automatization of the IHC protocols for CD73 detection as well as the quantification grid have been cross-validated between the partners (***D7.5, D2.8***). A major investment was performed by *CLB, UTU* and *CHUV partners* to analyze CD73 expression on multiple

tumor types using either Tissue multi array (TMA) or full sections according to the available tumor material and the size of the cohort to be analyzed. Retrospective tumor collections with clinical annotations have been defined in different partners' hospital institutions and IHC studies have been done on different tumor types including Melanoma, Lung, Breast, Ovarian, Bladder, Prostate, Head and Neck (H&N), hepatobiliopancreatic system and thyroid carcinoma as well as different subtypes of Lymphoma, all with unmet medical needs.

Overall, we observe variable expression on tumor cells according to pathologies ranging from few percentages of cases (breast tumors, lymphoma) to 100% of cases being positive (some hepatobiliopancreatic tumors). Cancers showing the highest rate of CD73 expression are represented by the hepatocellular and pancreatic ductal malignancies, lung, bladder, H&N carcinoma and melanoma. In most instances, the expression on tumor cells is focal and appears in several pathologies to be associated with the invasive front (lung, some thyroid carcinomas, melanoma) (***D2.8***). Beside expression on tumor cells, blood vessels express CD73 in most tumors, as well as mesenchymal stromal cells. CD73 expression on immune cells is also observed in most tumors at variable levels from few percentages up to >50% depending on tumor type (***D2.10***).

Clinical correlations have been performed when the percentage of expression was significant and clinical follow-up sufficient. In certain tumor types CD73 expression on tumor cells is of bad prognosis, while in others CD73 expression is of good prognosis. The expression on stromal cell and immune cells is in some instance associated with good prognosis, but this might be linked to the stromal cells and immune cell types themselves as CD73 expression varies according to different infiltrating cell types.
Thus, based on tumor cell expression and outcome, some tumor types are considered as potential primary tumor targets. Of note CD73 IHC might be of help in the diagnosis of some cases of pancreas and thyroid tumors difficult to interpret by anatomo-pathologists. In parallel, **a proprietary anti-huCD73 has been generated and selected for potential future development of an IHC companion diagnostic** (***D2.9***).

Furthermore, *UTU partner* established a standardized test format for subsequent large-scale analysis to quantify in biological fluids soluble CD73 (sCD73) by ELISA and demonstrated the strong correlation with the enzymatic activity (***D2.4***). This tool has been used to measure sera concentrations of sCD73 in the FinRisk 2002 cohort (1369 men, 1426 women). Prevalent or incident cancers were diagnosed in 358 individuals (12,8%) but no difference in sCD73 serum concentrations was observed between individuals with and without cancer. However, among patients with prevalent cancer, those who died had statistically higher sCD73 values than those who survived. (***D2.4***). Based on these results we believe that sCD73 alone may not have additive value in finding the right individuals for clinical trials or to follow up their treatment response. However, the feasibility of combining sCD73 measurements with other parameters have also been investigated and analyses are ongoing (***D7.4***).

Relevance of CD73 biology as an immune checkpoint in human

Focusing on understanding CD73 biology within T cell populations, *CLB* and *LICR partners* demonstrated that, in contrast to murine cells, CD39 and CD73 are poorly co-expressed within human T cell subsets. CD39 is selectively expressed on Tregs (suppressive T cells) and activated T cells whereas CD73 is expressed on a discrete $CD4^+$ memory subset (helper T cells) and on all naïve $CD8^+$ T cells

(cytotoxic T cells). On CD8$^+$ T cells this expression decrease during the differentiation process (***D3.3, D7.1***). On CD4$^+$ T cells, CD73 characterizes a memory/effector T cell population (Teffs) of Th1.17 profile producing both IFNγ and IL-17 at high levels (potent effector). CD73 renders this CD4 population highly sensitive to suppression by AMP and by ATP in presence of CD39$^+$ Tregs (***D3.3, D3.1, D7.1***). Of interest, Ado suppresses most cytokine production by T cells except IL-17 and IL-22. Our current model is that CD73 expression on this potent CD4$^+$ Teffs limits its reactivity in an inflammatory environment rich in ATP and that CD39$^+$ Tregs transform Th1.17 potent anti-tumor effector into "Th17 only" with pro-tumoral activity. Moreover, their expression of functional MDR1, the multidrug resistance protein 1, render them resistant to chemotherapy *in vitro* (***D3.1***) suggesting their capacity to resist to chemotherapy regimen *in vivo* that could contribute to the anti-tumor immune response. Finally, their lack of other inhibitory immune check-points (ICPi, PD1, CTLA-4, TIGIT, TIM3) (***D3.3, D7.1***) suggest that CD73 play a dominant role as an ICPi able to regulate the function of these unique Teffs.

In summary these results highlight a u**nique biology of CD73 regulating a potent anti-tumor effector**, and suggest **CD73 as a potential resistance mechanisms to anti-PD1/CTLA4 treatment**.

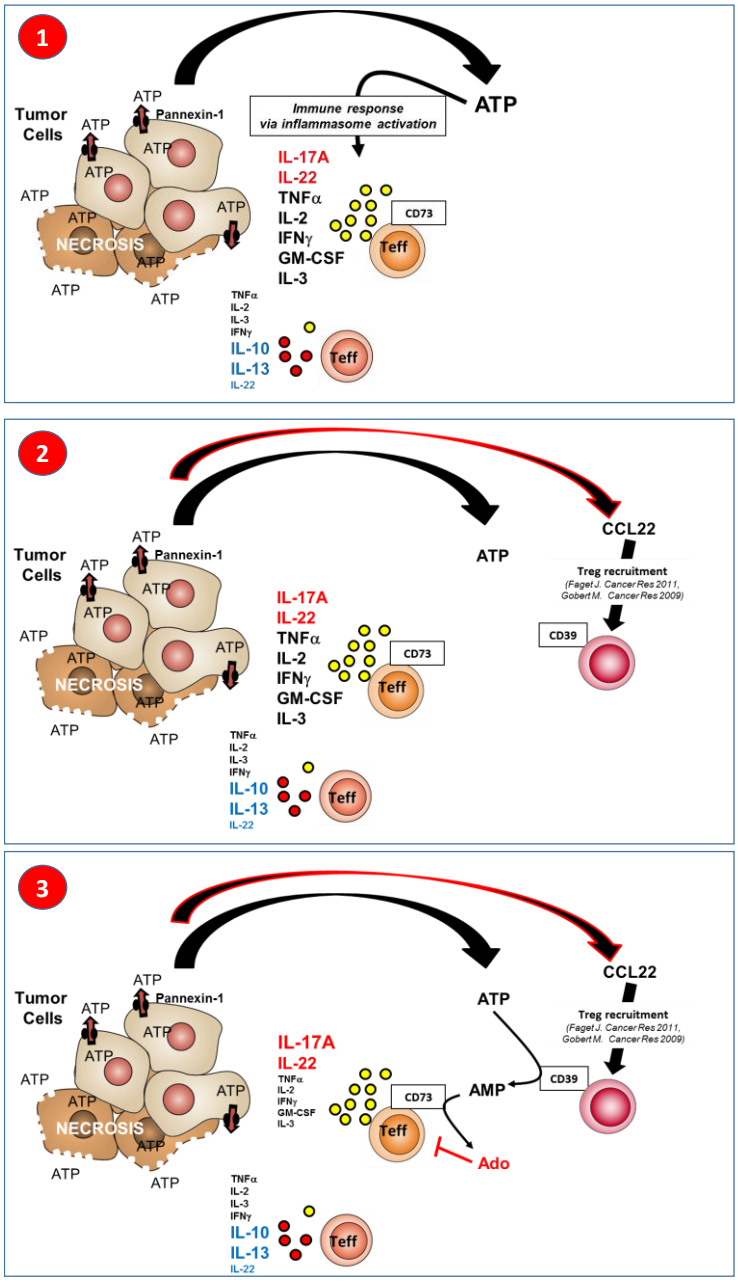

Figure 3: Autocrine Adenosine regulates tumor polyfunctional CD73+CD4+ effector T cells devoid of immune checkpoints: 1- ATP through inflammasome activation favors Th1.17; 2- Inflammation leads to CCL22 secretion and Treg recruitment ; 3- CD39high activated Tregs will target CD73+ Teffs through Ado production transforming potent Th1.17 anti-tumor effectors into protumoral Th17 T cells

LICR partner also evaluated the role of Ado receptors (ADORAs) in mediating T and NK cells immunosuppression. Overall, the receptor mainly expressed by immune cells on peripheral PBMC is A2A. We show that Ado immunosuppressive effects on human T cells are primarily exerted through A2A and that $CD8^+$ T_{CM} cells are more sensitive than the other $CD8^+$ T cell memory subsets since they express higher A2A levels (***D3.5***). In addition, TILs expanded from distinct tumor types also preferentially express A2A and have a similar sensitivity to Ado compared to peripheral T cells. Triggering of A2A in T cells increased PKA activation resulting in impaired mTORC1 (but not mTORC2) pathway, cytokine production and metabolic fitness. Our findings unveil the A2A/PKA/mTORC1 pathway as the main axis for Ado to impair T cell function and metabolic fitness (***D3.5***). Finally, NK cell function was also impaired by Ado. However, both A2A and A2B but also receptor-independent mechanisms may play a role in mediating these effects on this population (***D3.8***).

In summary these data identify **A2a as a dominant receptor mediating Ado suppressive activity on cytotoxic effectors** (CD8 T cells and NK) **through inhibition** of the mTOR pathway involved in **metabolic adaptation of the T cells in the unfavorable tumor environment**.

In this WP, the role of CD73 on tumor cells was also investigated in various assays by *UTU* and *CLB partners*. The most consistent observation relates to a role of CD73 in tumor invasion in 3D culture models (***D3.6***). Thus targeting CD73 may have additional benefit by preventing tumor cell invasion.

Furthermore, *CLB partner* also analyzed the regulation of CD73 expression (**D3.2**) and its implication during the migration of cells associated with tumor invasiveness (epithelial to mesenchymal transition, or EMT process (**D3.7**). Clear association between EMT and CD73 expression was made on TNBC human tumors and extended in carcinosarcoma of the breast (**D3.7**). This observation was also confirmed in a plastic mouse breast model undergoing EMT in vivo under immune pressure (**D3.7**). Together with the IHC observation that CD73 is focally expressed at the invasive front of certain tumors (**D2.8**), these data suggest a **role of CD73 in the invasive properties of CD73+ tumor cells, and that the immunosuppressive production of Ado may contribute in some tumor-types to increase tumor dissemination properties.**

Altogether these biological observations strengthen the importance of CD73 biology as an immune checkpoint in human, and validate its enzymatic neutralization as a therapeutic objective.

Development of neutralizing anti-huCD73 mAbs.

The generation and characterization of innovative and potent humanized CD73 neutralizing mAbs has represented a major collective effort. Monoclonal Abs were produced, purified and evaluated in various *in vitro* efficacy assays (affinity, specificity, inhibition of enzymatic activity, inhibition of Adenosine-mediated effect on immune cells, through degradation of AMP into Adenosine), using different cellular models (***D4.1, D4.2***). A group of anti-huCD73 Abs initially selected were further characterized (epitope mapping and mechanism of action (MOA)) (***D4.3***). One lead candidate was selected and has been successfully humanized (***D4.4, D4.6***).

In the course of the project, two pharmaceutical companies have developed their own blocking anti-CD73 Abs and they have initiated phase I clinical trials, Medimmune in 2015 and BMS in 2016.

Based on patent information, the two competitors Abs were produced and compared *in vitro* to the selected lead anti-huCD73 Ab candidate. The latter has distinct properties and epitope from the ones of the competitors (MedImmune, BMS), and better efficacy in several *in vitro* assays.

In parallel, surrogate anti-moCD73 blocking Abs were generated for use in syngeneic murine tumor models (an immunocompetent model for immunotherapy assessment) (***D4.5, D4.7***). *In vivo* efficacy experiments are underway.

New preclinical models for relevant in vivo therapeutic evaluation.

A major investment has been made by *the partners* to develop mouse models for *in vivo* evaluation of anti-CD73 mAbs (**Table 1**): i) syngeneic mouse tumor models expressing moCD73, ii) syngeneic mouse tumor models deleted for moCD73 (moCD73^{KO} ID8 cell line) (***D5.1***), iii) transplantable syngeneic mouse tumor models expressing huCD73 (huCD73 transfection in moCD73^{KO} ID8 and B16F10 cells) (***D5.2***), iv) generation of hCD73 KI mouse strain (***D5.3***)*;* v) transplantable xenogeneic human tumor cell lines (huCD73^{WT} and huCD73^{KO}) (***D5.4***), vi) transplantable xenogeneic huCD73 human tumor cell lines in immuno-deficient mice reconstituted with human immune cells (***D5.6***).

Type of models	Tumor cell lines	Main interest/objective	Limitations
syngeneic mouse tumor models expressing moCD73	B16F10 Melanoma, ID8-agg Ovarian, LLC-1 & SV-2*Lung, 4T1 Mammary, CT26 Colon carcinomas	Analyse immune response in immuno competent mice	Will not allow evaluation of anti-hCD73 not cross-reacting with mCD73
syngeneic mouse tumor models deleted for moCD73	ID8-agg Ovarian	Evaluate the importance of mCD73 expressed on tumor cells	
transplantable syngeneic mouse tumor models expressing huCD73 (huCD73 transfection in moCD73^{KO} ID8 and B16F10 cells)	B16F10 Melanoma, ID8-agg Ovarian	The replacement of mCD73 by hCD73 allows to evaluate the therapeutic anti-hCD73 drug candidate, even in wt mice	Issue with immunogenicity of hCD73 in wt mice
generation of hCD73 KI mouse strain		Allow to evaluate the targeting of hCD73 expressed on host cells (the tumor cell being hCD73 pos or neg)	If ki functional, this is a perfect model to use with hCD73 expressing mouse tumor cell lines
transplantable xenogeneic human tumor cell lines (huCD73^{WT} and huCD73^{KO})	HCT116 & HT29 & Isreco-1 Colorectal, NCI-H292 (huCD73^{WT} and huCD73^{KO}) lung, MDA-MB-231 Breast, MIA PaCa-2 Pancreatic carcinoma	Allow to target hCD73 expressed on human xenogenic tumor cell line in immunodeficeint mice	The absence of adaptive immune system prevent to evaluate the immunomodulatory effect of hCD73 neutralisation
transplantable xenogeneic huCD73 human tumor cell lines in immuno-deficient mice reconstituted with human immune cells	EBV LCL B cell lymphoma cell line CD73+	The model allow to target hCD73 expressed on tumor cells or human immune cells	The models are complex, heavy and difficult to standardize, and with a short window of experiments due to to xeno-GVH

Table 1: Mouse models for *in vivo* evaluation of anti-CD73 mAbs

The TumAdoR consortium had to counteract numerous technical difficulties to assess the efficacy of the lead candidate due to 1) immunogenicity of huCD73 that led to the loss of huCD73 on transfected tumor cells (ID8 and B16 models) (***D5.2***), and 2) the absence of in vivo validation of the huCD73 Knock-In mouse model generated by *Polygene partner* (***D5.3***) that prevents for the moment the evaluation of the *in vivo* efficacy of the drug candidate. Indeed, for unknown reason the KI construct was expressed at the mRNA level but the human CD73 protein could not be detected in vivo in the KI mice (western blot and Immunofluorescence).
The use by *CLB partner* of transplantable xenogeneic human tumor cell lines in SCID mice did not demonstrate clear activity of anti-huCD73 blocking Ab, probably due to lack of efficient immune system in these models (***D5.4***). As therapeutic anti-huCD73 have been developed based on the immune regulatory role of CD73, we focused on models with human immune system. The last series of models developed by *UTHSCSA partner*, thus, consist of human tumors growing in immunodeficient mice reconstituted with autologous human peripheral blood cells. These models being complex to set-up and not standardized, their use within EU-funded project lifetime has been challenging. For several reasons (early GVHD, unstandardized tumor growth) the experiments performed in the humanized NSG mice implanted with the human tumor models did not allow yet to reach a conclusion regarding the activity of the anti-huCD73 mAb candidates (***D5.6***).
An alternative plan is ongoing by *Polygene Partner* to generate huCD73 transgenic (huCD73tg) mice under the control of other promotors to have expression of huCD73 respectively in T/B lymphocytes and in endothelium (cells normally expressing CD73 in human and mice) to evaluate activity of a blocking anti-CD73 Ab. In these mice, tumor cell lines expressing huCD73 will not be rejected as huCD73tg mice will be tolerant to huCD73 Although the transgenic mice are not available at the end of the project, they will be used as soon as possible to investigate *in vivo* the impact of the anti-huCD73 blocking Ab

Finally, a massive collective effort was done between *IPH*, *LICR* and *CLB partners* to explore, in therapeutic setting, the impact of moCD73 neutralization in multiple purely syngeneic models (**Table 2**) using several surrogate mAbs. Combination with anti-PD1/L1 mAbs were also performed in several of these models. Experiments are still ongoing.

Cell line	Tumor model	Injection site	Mouse Strain	moCD73 expression	Immunotherapy response to PD-1/PD-L1 blockade
B16F10	Melanoma	Sub-cutaneous	C57BL/6	Negative	None
ID8-agg	Ovarian carcinoma	Intra-peritoneal	C57BL/6	Positive	None
LLC-1	Lung carcinoma	Sub-cutaneous	C57BL/6	Negative	Partial
SV-2*	Lung carcinoma	Sub-cutaneous	C57BL/6	Positive	None
4T1	Mammary carcinoma	Sub-cutaneous	BALB/c	High	Partial
CT26	Colon carcinoma	Sub-cutaneous	BALB/c	Negative	Partial

Table 2 : Different syngeneic tumor models developed to investigate the impact of neutralizing anti CD73 mAbs.

Development of specific assays for toxicity evaluation.

For the validation of the interest of CD73-based therapeutic approach to treat cancer, it is key to verify the absence of safety issues related to CD73 blockade. Assays to evaluate toxicity on blood vessel integrity, vascular leakage, and immune response to test antigens have been established and performed in $CD73^{KO}$ mice by *UTU partner*. huCD73 expression on frozen normal human tissues using the lead anti-huCD73 mAb has been performed by *IPH partner*. No safety issues have been observed (**Table 3**).

Assays	CD73KO	CD73 small molecule inhibitors in wt mice	Anti-huCD73 mAb in wt mice	Anti-huCD73 candidate in Cynomolgus	Anti-hCD73 in in vitro assay	Anti-huCD73 candidate on healthy human tissues
Blood vessel integrity	No leakage	No leakage	ND	Ongoing	No leakage	ND
Immune response to test antigens	Normal response	Normal response	ND	ND	ND	ND
Pathological evaluation	No abnormality	ND	ND	Ongoing	ND	ND
Cross-Reactivity on healthy human tissues	ND	ND	ND	ND	ND	No aberrant cross-reactivity

Table 3: In vitro and in vivo assays to evaluate side effects and off target effects of anti-hCD73.

Due to absence of huCD73 KI mice, the evaluation of the safety/toxicity of the lead candidate has not been performed in mice. As an alternative approach, a novel and potent inhibitor of CD73 enzymatic activity was used in $CD73^{WT}$ mice by *UTU partner*. These experiments did not reveal any increased vascular leakiness, suggesting that inhibition of the enzymatic activity of CD73 with antibodies should not lead to serious side effects i.e suggesting that the new therapy targeting CD73 will be safe.

IPH partner further evaluated the lead anti-huCD73 mAb candidate antibody on frozen healthy tissue TMA containing 15 types of normal organs each. A membranous/cytoplasmic expression on endothelial cells and lymphocytes in lymphoid organs and tissues, as well as on cells from immune privileged sites was mainly observed, in agreement with the literature on CD73 expression.

As the lead candidate cross-reacted with cynomolgus CD73, a preliminary evaluation of safety was done in cynomolgus. The experimental part of the study has been performed, complete analysis will be available after the project end.

From the current TumAdoR consortium current data **no toxicity issues are expected following CD73 enzymatic activity neutralisation in clinic**.

In the frame of TumAdoR project a series of imaging tools allowing the longitudinal monitoring of tumor growth in vivo has been developed by the partners (luciferase/bioluminescence/NightOWL by *UTHSCSA and CLB partners*, Quantum FX Low Dose Computed Tomograph by *CLB and LICR partners*). These tools will allow in the future to reduce drastically the number of animals used (by 50 to 80% in spontaneous tumor models), through early identification of tumor development and constitution of homogenous experimental group of mice. Furthermore, these imaging tools will allow a longitudinal follow-up of mice under treatment, that would require 10 time many mice in conventional tumor growth evaluation (mice sacrifice at each time point), in particular for deep tumors (Lung and ovarian/pancreas models).

Thus the imaging tools develop during the TumAdoR consortium will allow a major reduction in the number of mice needed for longitudinal follow-up of mice under treatment.

Development of new Immuno-monitoring tools.

Specific tools to assess target expression and to monitor relevant immune parameters in patients during the treatment have been developed by *LICR* and *CLB partners* (Table 4). Multi-parametric flow cytometry tools have been setup to analyse impact on differentiation/ polarization of T cells subsets and activation status of B cells, NK cells, DCs, monocytes in combination with CD73 and other biomarkers expression (***D7.1***).

Parameters	Assays	Vadidation in heathy donors	SOP transfer in the consortium	Evalua-tion in patients	Compatibility with anti-CD73 drug candidate
Multi-parametric flow cytometry tools for CD73 and CD39 on immune cell subsets and activation status	-differentiation/ polarization of T cells	done	done	done	Yes
	- immune subsets and activation status	done	done	done	Yes
	- B cells, NK cells, DCs, monocytes	done	done	done	Yes
TAA-specific immune responses	- fluorescent bare-coding MHC/antigen peptide multimers multiparametric	done	done	done	Yes
	- flow cytometry analyses by intracytoplasmic cytokines staining	done	done	done	Yes
sCD73 in plasma	- Elisa	done	done	done	Yes
	- enzymatic activity of sCD73	done	done	done	Yes
CD73 expression in situ	IHC	done	done	done	Yes
CD73 and immune cells in situ	Multi-IF	ND	ND	done	Yes

Table 4: Immunomonitoring tools to evaluate immune parameters during anti-hCD73 clinical trial.

To measure TAA-specific immune responses, two experimental approaches have been pursued: (i) fluorescent bare-coding MHC/antigen peptide multimers for antigen specific T cell monitoring and (***D7.2***) (ii) multiparametric flow cytometry analyses by intracytoplasmic cytokines staining following TCR (T cell receptor)-mediated T cell activation. Flow cytometry strategies designed to follow specific T cell responses can be applied to any antigen of choice depending on the tumor targeted. This work was also completed by the development of tools to assess the expression of CD73 during drug treatment in case the drug candidate competes with the commercial antibodies.

An assay to monitor the enzymatic activity of sCD73 in plasma has been setup. Furthermore, a multiplex assay allowing the concomitant analysis of 48 analytes (including cytokine/chemokine/growth factors) have been successfully evaluated in plasma from cancer patients. These potentially will serve as new biomarkers for the patients selected for and/or treated with anti-CD73 antibody therapy in the future.

Overall, the work performed during TumAdoR project allowed to generate comprehensive knowledge of CD73 and CD39 expression in the main peripheral blood immune cell subsets and of sCD73 enzymatic activity in cell-free human serum.
The generated tools used could be determinant for future immunomonitoring in clinical trial and the information generated could represent important normal baseline value for patient comparison and follow up.
Overall, efforts put in this consortium to develop and harmonize immunomonitoring tools and standard operating procedures (flow cytometry panels, samples collection/handling, CD73 IHC protocols and reading grids) could be **very relevant to speed up validation of the strategy to apply in clinical samples obtained during the future clinical trial**.

Final TumAdoR results

Although we have experienced delay in ***WP2*** and ***WP5*** (technical difficulties, absence of functional huCD73 KI mice), the program has progressed very well with the development and selection of the lead candidate for a neutralizing anti huCD73 mAb (***WP4***). *IPH partner* has selected the final lead candidate of humanized anti-huCD73 Ab, and produced large batches of different Abs (***WP4***), for *in vivo* efficacy (***WP5***) and safety evaluation (***WP6***) by *IPH, LICR, CLB* and *UTHSCSA partners*. *IPH partner* has further characterized the lead candidate, and generated back-up anti-huCD73 Abs. Many novel data on huCD73 biology on $CD4^+$ T cells and A2A functionality on $CD8^+$ T cells and NK cells have been generated, increasing the relevance of CD73 therapeutic targeting (***WP3***). Current data also indicate lack of safety issues (***WP6***). Furthermore, standardized tools for IHC (automated protocol and quantification grid) have been developed for the analysis of CD73 target expression on large cohorts of human solid tumors (***WP2***). *CHUV*, *CLB* and *UTU partners* have generated knowledge on CD73 distribution in human selected tumors and its impact on tumor progression and patient survival allowing a comprehensive knowledge of CD73 expression that will be critical for the future orientation of the clinical development. A mAb has been generated, for use in future IHC companion diagnostic for anti-huCD73 treatment *(**WP2**).* A major investment has been made by *the partners* to develop mouse models for *in vivo* evaluation of anti-CD73 mAbs, despites numerous technical difficulties (immunogenicity of huCD73 in wt mice, absence of in vivo validation of the huCD73 Knock-In mouse model), the consortium select relevant models to assess CD73 biology in vivo

and the impact of the drugs on immune parameters (***WP5***). Finally, tools for the immune-monitoring of the future clinical trial with the drug candidate are now all in place (**WP7**).

Overall, with the exception of the *in vivo* proof of activity, the program has reached most of its objective albeit with an overall 6-months delay.

In conclusion, the TumAdoR project, thanks to the complementary expertise and objectives of the partners, has generated:

- New knowledge on CD73 as an ICP blocker on $CD4^+$ Teffs,
- New knowledge on A2A and A2B expression, function and signaling on $CD8^+$ T cells and NK cells
- New in vitro assays to evaluate drug candidates blocking CD73 pathway
- New preclinical models to assess efficacy of this strategy, ,
- Humanized efficient neutralizing anti-huCD73 mAbs, with higher efficacy in vitro than competitors and differentiated MOA
- Tools for future companion diagnostic IHC to assess CD73 expression
- Comprehensive knowledge of huCD73 expression in human solid tumors that will be critical for the future orientation of the clinical development and first clinical trial
- Immuno-monitoring tools to follow relevant immune parameters in patients under anti-CD73 therapy to assess biological efficacy and investigate on and off targets.

The **Table 5** below list all deliverables of the project.

Deliverable n°	Deliverable name
D1.1	Minutes of the official meetings
D1.2	Collaborative tool
D1.3	Written feedback from ethical and scientific advisory board
D2.1	Ethical notifications consolidating inputs from WP2 to 7 regarding ethical approvals/opinions /notifications by the competent legal local/national Ethics Boards/Bodies/administrations
D2.2	Establishing of a sample biobank and database for analysis
D2.3	Software and storage informatic system for data acquisition, analysis and sharing among partners
D2.4	Validation of sCD73 ELISA as a relevant biomarker of CD73 expression in the tumor microenvironment
D2.5 §	Validation of high throughput minute input ELISA
D2.6	Validation of IHC CD73 and other biomarkers to quantify expression in the tumor microenvironment
D2.7	Validation of gene expression of relevant biomarker candidates
D2.8	Identification of the tumor pathology with the optimal target expression for the first clinical trial
D2.9 *	Selection and pre-validation of an anti-CD73 mAb, to be used in a future companion diagnostic to determine target expression and patient segregation
D2.10	Annotation of CD73, associated biomarkers and immune cells to clinical features and biology of certain tumors
D3.1	Understanding of CD73 and CD39 expressing cell cooperation in the suppressive tumor microenvironment of the targeted human tumors
D3.2	Identification of key regulators of CD73 expression on tumor cells and its microenvironment
D3.3	Characterization of the unique features of CD73+ effector T cell subset as a key target of suppressive CD39+ Tregs
D3.4 §	Elucidation of the CD73-dependent trafficking of immune cells
D3.5	Identification of the AdoR contributing to the Ado immuno-suppressive activity
D3.6	Determination for the TumAdoR selected pathologies of the impact of CD73/Ado on human tumor cell proliferation and migration in vitro
D3.7	Demonstration of the functional connection between the EMT process and CD73 expression on tumor cells
D3.8	Identification of the adenosine mediated effects and of the AdoR contributing to these effects in NK cells

Deliverable n°	Deliverable name
D4.1	Finalisation of the target product profile
D4.2	Biological tools and robust biological assays for mAbs characterization
D4.3	Production of few mgs of chimeric Abs for in vitro 2ndary screenings
D4.4	New and innovative neutralizing anti-huCD73 Abs
D4.5	Production of 1g of 5 chimeric Ab candidates for in vivo studies
D4.6	Humanization of lead Ab candidate
D4.7	Production of 1g of humanized Lead Candidate for pre-clinical toxicity and PK/PD studies
D5.1	Validation of transplantable tumor models to evaluate **cross-reactive** anti-mouse CD73
D5.2	Validation of transplantable tumor models to evaluate non cross-reactive anti-human CD73 antibodies
D5.3	Generation of the humanized CD73 KI mouse
D5.4	Validation of xenogeneic human tumor models in immunodeficient mice with or without human immune reconstitution
D5.5	Identification of optimal novel screening models to test the efficacy of anti-hCD73 in vivo
D5.6	Evaluation of therapeutic impact of human CD73 neutralization in transplantable tumor models
D5.7 §	Evaluation of therapeutic impact of mouseCD73 neutralization in spontaneous mouse tumor models
D5.8	Evaluation of the anti-immune response triggered by CD73 using anti-CD73mAbs
D5.9	Selection of the anti-hCD73 lead candidate based on in vivo anti-tumoral therapeutic activity
D6.1	Normal Tissue Distribution for CD73 in human tissue
D6.2	Demonstration of the absence of side effect on vascular permeability
D6.3	Demonstration of the safety profile of targeting CD73 with an antibody in mouse
D6.4	Expression profile of the selected human mAbs neutralizing hCD73 enzymatic activity
D6.5	Demonstration of the safety profile of the selected lead candidate mAbs neutralizing hCD73 enzymatic activity
D6.6 *	Preliminary PK and toxicity for the lead mAb candidate in NHP
D6.7	Development of in vivo imaging of tumor progression for ethical reasons
D7.1	Validation of a set of standardized flow cytometry tools to monitor immune cell competence in patients undergoing anti-CD73 treatment
D7.2	Validation of flow cytometry based assay to monitor tumor antigen specific T cell responses in patients undergoing anti-CD73 treatment
D7.3	Novel assays to monitor on-target and off-targets effects of antibody mediated CD73 blockade
D7.4	Validation of bead array-based multiplex assay to measure immune analytes in plasma from patients
D7.5	Harmonized immune monitoring assays to be integrated in the phase I clinical trials of antibody mediated CD73 blockade
D8.1	Draft and final plan the use and dissemination of foreground
D8.2 §	Report of patient requests
D8.3	Report and associated material of the training activities, training sessions
D8.4	Report on contribution to standards
D8.5	Project identity set (logo, brochure…)
D8.6	Project website
D8.7	Software and storage informatic system for data analysis and sharing among partners

Table 5: Complete list of TumAdoR deliverables. §: deliverables removed during the project through Amendment process; * : Deliverable name changed here compared to Annex 1 to better reflect their achievement at this stage in the frame of putative therapeutic development.

1.4 Potential Impact and Dissemination activities and exploitation of results

The TumAdoR project allowed the transfer of knowledge and know-how between the partners, and to create synergies to accelerate research and development of innovative mAbs for potential new anti-cancer treatment. Tremendous amount of new data are available already available on CD73 expression in tumors and on CD73 biology and it's role in the CD73/Ado/ADORs axis in immunosuppression and tumor progression. Besides, the project allowed the generation of many new experimental models that will facilitate future research with strong *in vitro* or *in vivo* relevance, and optimize animal use in pre-clinical steps.

The results obtained in the course of TumAdoR have been presented to date at several scientific events (14 posters, 8 oral presentations) and described in peer-reviewed publications (to date in 3 published papers and 1 Thesis; 8 papers in preparation including 1 in revision).

Information regarding flow cytometry panels, SOPs for samples collection/handling and screening of huCD73 expression on FFPE-embedded tumors by IHC have been generated and distributed to all partners for harmonization purposes. They will be disseminated to the scientific community with the several peer-review publications arising from TumAdoR project.

For the moment, 4 patents arose from the project and will allow exploitation of the results of the consortium. Futher research is still needed before the main results are available for the patients, but in case the clinical Phase I assay is promising, the impact on public health would represent a lot of hope for patients with cancer without specific treatment to date. The mAbs oncology market is constantly growing and the economic impact of the TumAdoR anti-CD73 mAb could be comparable to previous successful new mAbs, in range of several hundred millions Euros.

In addition, TumAdoR project had important impact in the training of students and young researchers in the field of immunotherapies. Members of the TumAdoR consortium contributed to disseminate knowledge on immunotherapy and immune checkpoint role/function and treatment through multiple seminars in institution or meetings and through courses for master and MD students (total audience represent more than 1000 students). One PhD thesis was defended in direct linked with the project, and one more is planned for September 2018. Finally, communication to larger public was also made *via* project website, press releases and partner's websites.

1.5 Consortium and contact information

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