TUMOR INFILTRATING LYMPHOCYTES AS A BIOMARKER OF RESPONSE FOR CTLA-4 TARGETING THERAPIES

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 # B93

Abstract

Immunotherapy based on monoclonal antibodies (mAbs) targeting cancer cells is now developed as a valid approach to treat cancer. Suppressive mechanisms in immune responses normally play a critical role in maintaining immune homeostasis. However, these suppressive mechanisms are also considered as one of the main reasons for the failure of cancer immunotherapies because they induce peripheral tolerance of tumor-specific immune responses and allow tumor growth. Regulatory T cells have been revealed as the most important population of immune suppressors, and their depletion has been reported to enhance antitumor immune responses.

CTLA-4 was reported as a critical target for regulatory T cell function and thus blockade of CTLA-4-mediated signals has been suggested as a possible strategy to treat cancers. The first anti-CTLA-4 human mAb, ipilimumab, was approved in 2011 by the FDA for use in metastatic melanoma. Success for ipilimumab was reported in a large phase III clinical trial involving patients with metastatic melanoma, who had undergone previous failed treatment.

Mice obtained from Charles River (France) were orthotopically or subcutaneously injected with syngenic tumor cell lines. They received repeated intraperitoneal injections of antibody directed against CTLA-4. During the course of the experiment, animals were terminated under anesthesia when they displayed significant signs of physiological changes. Animal housing and experimental procedures were performed according to the French and European Regulations and NRC Guide for the Care and Use of Laboratory Animals. Animal facility is authorized by the French authorities (Agreement N°B21231011EA).

The tumor and tumor draining lymph nodes were collected for subsequent FACS analysis to study the immune response in mice. Cells suspensions were prepared from tissues either by mechanistic dissociation or by enzymatic digestion. The antigens associated antibodies used for FACS analyses were CD45, CD3 and CD8 for effector T cell lymphocytes, and were CD45, CD3, CD4, FoxP3 and CD25 for regulatory T cell lymphocytes. The stained cells were analyzed with a LSR II flow cytometer (BD Biosciences) equipped with 3 excitation lasers at wavelengths 405, 488 and 633nm.

In CT26 and EMT6 models, an increase in T effector *vs* T regulator infiltrating immune cells ratio was observed for responding mice treated with CTLA-4 mAb. In 4T1 model, no ratio change was observed for mice treated with CTLA-4 mAb.

Pending new generation of humanized mouse models, the growing interest in immunology as a cancer therapy shows the limitation of conventional xenograft models in immunodeficient animals. A more effective approach is the use of syngeneic mouse models.

We here report on syngenic tumor models our capacity to identify biomarkers of response to CTLA-4 targeting therapies using detailed analysis of tumor immune infiltrating cells.

Material and Methods

In vivo experiments

Immunocompetent mice were obtained from Charles River (France). Animals were orthotopically (OT) or subcutaneously (SC) injected with syngenic tumor cell lines on D0. The animals received repeated injections of antibodies directed against CTLA-4. Isoflurane was used to anaesthetize the animals before cells injection and termination. All logistical parameters of the study (dosing, collection, measurements, raw data, lethality, behavior and results of autopsy…) were managed using Vivo Manager software (Biosystemes, Dijon, France). During the course of the experiment, animals were sacrificed under anesthesia when they displayed significant signs of physiological changes. Animal housing and experimental procedures were realized according to the French and European Regulations and NRC Guide for the Care and Use of Laboratory Animals. Animal facility is authorized by the French authorities (Agreement N° B21231011EA). All procedures using animals were submitted to the Animal Care and Use Committee of Oncodesign (Oncomet) agreed by French authorities (CNREEA agreement N° 91) [1, 2, 3].

Immune cell detection in mice tissues

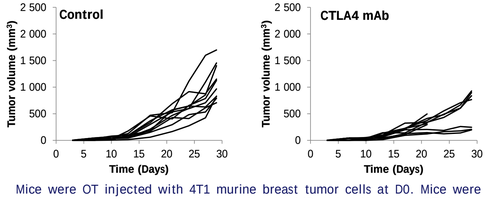
The tumor and tumor draining lymph nodes were collected for subsequent FACS analysis to study the immune response in mice. Cells suspensions were prepared from tissues either by mechanistic dissociation or by enzymatic digestion. The antigens associated antibodies used for FACS analyses are described in Table on right. The stained cells were analyzed with a LSR II flow cytometer (BD Biosciences) equipped with 3 excitation lasers at wavelengths 405, 488 and 633 nm.

Panels (up to 10 colors)	populations	markers
CD45, CD3, CD4, CD8, CD25, FoxP3	**Teff**	CD45+ CD3+ CD4- CD8+
	Treg	CD45+ CD3+ CD4+ CD8- (CD25+) FoxP3 +
CD45, CD3, CD8, TNFα, Perforin, Granzyme B	**Teff**	CD45+ CD3+ CD8+ (TNFα, Perforin, Granzyme B)
CD45, CD3, CD11b, Gr1, Ly6G, Ly6C, iNOS, Arg1	**Total MDSCs**	CD45+ CD3- CD11b+ Gr1+
	M-MDSCs	CD45+ CD3- CD11b+ Gr1+ Ly6G- Ly6C+ (iNOS, Arg1)
	G-MDSCs	CD45+ CD3- CD11b+ Gr1+ Ly6G+ Ly6C- (iNOS, Arg1)
CD45, CD3, CD11b, Gr1, CD68, CD80, CD206	**Total TAMs**	CD45+ CD3- CD11b+ Gr1-
	M1 TAM	CD45+ CD3- CD11b+ Gr1- CD68+ CD206-
	M2 TAM	CD45+ CD3- CD11b+ Gr1- CD68- CD206+

1-Principe d'éthique de l'expérimentation animale, Directive n°2010/63 CEE du 22 septembre 2010, Décrêt n°2013-118 du 01 février 2013.
2-NRC Guide for the Care and Use of Laboratory Animals.
3-United Kingdom co-coordinating committee on cancer research guidelines for welfare of animals in experimental neoplasia, Br. J. Cancer 2010, 102: 1555-1577.

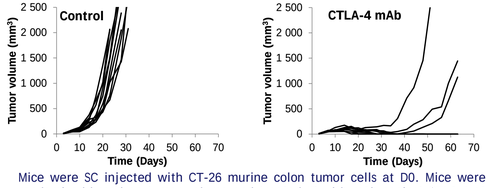
Results

4T1 Breast Carcinoma Model



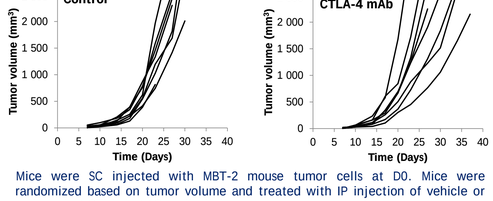
Mice were OT injected with 4T1 murine breast tumor cells at D0. Mice were randomized based on tumor volume and treated IP with mAb against CTLA-4 at 10 mg/kg/inj.

CT-26 Colon Carcinoma Model

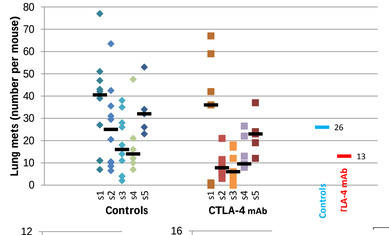


Mice were SC injected with CT-26 murine colon tumor cells at D0. Mice were randomized based on tumor volume and treated IP with mAb against CTLA-4 at 10 mg/kg/inj.

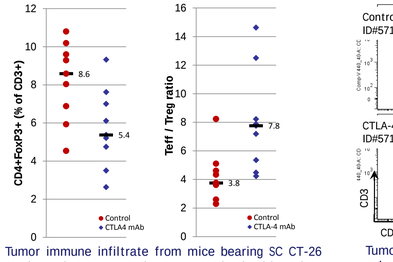
MBT-2 Bladder carcinoma Model



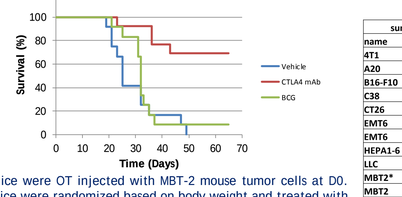
Mice were SC injected with MBT-2 mouse tumor cells at D0. Mice were randomized based on tumor volume and treated with IP injection of vehicle or mAb against CTLA-4 at 10 mg/kg/inj.



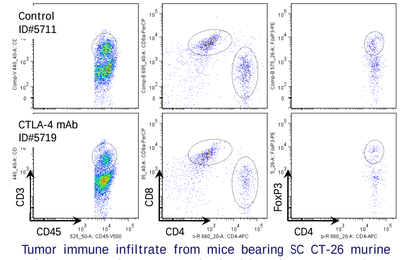
Lung metastasis number from mice bearing OT 4T1 murine breast tumors. Mice were randomized based on tumor volume and treated IP with mAb against CTLA-4 at 10 mg/kg/inj. Individual and median values are from 5 independent studies (s1 to s5). Median values from all studies are also presented.



Tumor immune infiltrate from mice bearing SC CT-26 murine colon tumors. Mice were randomized based on tumor volume and treated IP with mAb against CTLA-4 at 10 mg/kg/inj. Individual and median values for Treg population and Teff/Treg ratio.



Mice were OT injected with MBT-2 mouse tumor cells at D0. Mice were randomized based on body weight and treated with repeated intravesical instillations of BCG at 1.35 mg/kg/inj or treated with IP injection of mAb against CTLA-4 at 10 mg/kg/inj.

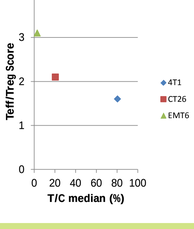


Tumor immune infiltrate from mice bearing SC CT-26 murine colon tumors. Mice were randomized based on tumor volume and treated IP with mAb against CTLA-4 at 10 mg/kg/inj. Representative FACS pictures.

Mouse immune checkpoint *in vivo* efficacy

summary		CTLA-4	
name	site	n	T/C (median)
4T1	**OT**	8	81
A20	**SC**	1	25
B16-F10	**SC**	2	100
C38	**SC**	1	11
CT26	**SC**	6	21
EMT6	**SC**	7	3
EMT6	**OT**	0	NA
HEPA1-6	**OT**	1	35
LLC	**SC**	1	97
MBT2*	**OT**	1	260**
MBT2	**SC**	1	83
Renca*	**OT**	1	100**

* Survival
** ILS



Conclusions and perspectives

- A large panel of syngeneic mouse models is available for evaluation of immune checkpoint inhibitors such as CTLA-4 targeting therapies.
- Among this panel, some tumor models are known as partial or non responders to CTLA-4 targeting therapies, allowing investigation of antitumor effect of combined treatments.
- Flow cytometry is the main method to evidence biomarkers of response to CTLA-4 targeting therapies and immunohistochemistry analyses would be used as complementary readout.
- Newly available RNA sequencing data will help to understand how genomics information could be used as biomarker of response for these new therapies.