White Paper

Syngeneic mouse models as a tool to study immune modulatory effects of cancer therapeutics

Immuno-Oncology Platform

Subcutaneous Models
Orthotopic Models
Metastasis Models

Flow Cytometry
Cell Sorting
Immuno-Histochemistry
Target Validation
Syngeneic mouse models as a tool to study immune modulatory effects of cancer therapeutics

Every cancer treatment has the potential to induce a stimulatory or inhibitory effect on the immune response to a tumor. Scientific knowledge about the significance of the immune system for tumor eradication during conventional treatment is growing quickly, and an increasing number of immune-modulating drugs are entering clinical trials for cancer treatment. This necessitates investigating these drugs in the presence of an intact immune system, and syngeneic tumor models are the ideal tool to achieve this. In addition to our many xenograft mouse models, we established several syngeneic tumor models, which we thoroughly characterized with respect to immune phenotyping and response to immune checkpoint inhibition. Four of them are introduced in this white paper.

Introduction
In a healthy body, the immune system puts consistent pressure on emerging tumor cells by efficiently eliminating cells that acquire malignant properties. However, if tumor cells defeat the body’s immune surveillance, they still face persistent immune pressure, which they oppose by immune evasion or even active immune suppression. Cancer treatment disrupts the pre-existing equilibrium between immune pressure and immune evasion in a patient’s tumor – irrespective of the choice of therapy. It has become increasingly evident that the success of many conventional drugs relies on their side effects to activate immune responses for tumor tissue eradication (L. Galluzzi et al. 2012). In contrast, immune therapeutics are designed to support the immune system in its struggle to eliminate tumor cells by breaking tolerance or converting the tumor’s environment from immune-suppressive to immune-activating. To evaluate potential treatments, drug testing in animals with a functional immune system is crucial and mandatory to meet current drug development standards (JP Hegmans et al. 2014). In this white paper we will introduce four syngeneic tumor models to evaluate the immune modulatory capacities of new cancer treatments. As an example, we will demonstrate how treatment against immune checkpoint PD-1/PD-L1 (programmed cell death protein 1/ligand 1) impacts tumor growth and alters immune cell populations.

Flow cytometry analysis of syngeneic tumor models

The four syngeneic models characterized in this white paper are
• the 4T1 breast tumor model,
• the B16-F10 melanoma model,
• the CT26 wt colorectal tumor model,
• and the RENCA kidney tumor model. All models except B16-F10 were implanted subcutaneously into the flank of the mice. B16-F10 cells were inserted orthotopically into the dermis of the mice. Mice were sacrificed approximately three weeks after cell implantation, the solid tumors were isolated and dissociated into single cell suspensions, and ana-

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*orthotopic implantation **intravenous injection
*Carcinoembryonic antigen suited for investigation of tumor-specific T cell response

Syngeneic tumor models

Syngeneic tumor models are created by engraftment of murine tumor tissue into wild type mice of the same strain. In contrast to xenograft models, the immune system in syngeneic mice is intact to facilitate investigation of all aspects of adaptive and innate immunity. The mouse strains of engrafted tissue and host tissue are matched to prevent graft versus host disease.
lysis of T cells, macrophages, and myeloid cell subsets was performed using flow cytometry (Figure 1).

The goals of immune phenotyping are to gain information about
a) which immune cells are present in the tumors,
b) how many of those immune cells are present, and
c) whether the amount of the immune cells correlates with the size of the tumors.

Our four tumor models differed in the total numbers of tumor-infiltrating immune cells (leukocyte populations) as well as the composition of individual immune cell subsets (Table 1). For example, 4T1 tumors showed the highest amount of infiltrating leukocytes and the immune cells were mainly PMN-MDSCs and neutrophils (please see the insert for information about the immune cell subsets). B16-F10 and CT26.wt tumors on the other hand were very poorly infiltrated by leukocytes. All tumors had a similar relative amount of M1 and M2 macrophages.

Figure 1. Gating strategy for flow cytometric analysis of tumor-infiltrating immune cell subsets.
(A) Immune cell populations in CT26.wt tumors were investigated three weeks after tumor implantation in the flank of the mouse. Tumors were isolated and dissociated into single-cell suspensions. Cells of one tumor that were stained with three antibody panels and analysed by flow cytometry are shown.
(B) For comparison, a picture of a 4T1 tumor is shown with a substantial amount of infiltrating PMN-MDSCs.

Syngeneic tumor models as tools in drug development

CAN BE used for
- Detecting immune modulatory molecules
- Mechanism of action analysis for characterizing immune-modulating effects
- Investigating side effects of conventional drugs on the immune system
- Combining conventional therapies with immune checkpoint inhibitors

CANNOT BE used for
- Evaluating human-specific antibodies and compounds
- Studying long-term therapy

ProQinase Immuno-Oncology Platform
- Seven validated syngeneic tumor models (subcutaneous, orthotopic and metastasis models are available)
- Full characterization of immune cell subsets via flow cytometry with and without PD-L1 blockade
- Target validation in tumor tissue or lysate
- Sorting of immune cell subsets
- Immuno-histochemistry
- Pick-up service of compounds from the United States
- Hollow Fiber Screening
In Vivo Testing Services

Immuno-Oncology Platform

A particular subset holds an immune-activating (tumor growth suppressing) or immune-suppressive (tumor growth supporting) role. Interestingly, the frequency of some immune cell subsets did indeed significantly correlate with tumor volume (Table 2). For example, we can assume that the growth of CT26.wt tumors is dependent on the presence and absence of M1 and M2 macrophages. Furthermore, B16-F10 tumors seem to rely on the presence of activated CD8+ T cells to overcome the suppressive function of regulatory T cells. Although B16-F10 tumors were infiltrated by M-MDSCs with the highest frequency, the correlation data suggest no important role of these cells for tumor growth.

Immune-checkpoint blockade via anti-PD-L1 antibody

A very attractive target for immune therapy against cancer is the immune checkpoint PD-1/PD-L1 since it is a mechanism tumor cells employ to suppress the activity and proliferation of tumor-infiltrating immune cells (A. Buqué et al. 2015). In addition, targeting this pathway interferes with communication between immune cells in lymphatic tissues and “takes the brake off” immune-activating processes.

All four tumor models were tested with an anti-PD-L1 antibody treatment. Of all tumors, only CT26.wt tumors responded to therapy with significant tumor growth inhibition (Figure 2). We evaluated the tumor infiltrating immune cells of treated and untreated mice via flow cytometry at the endpoint of the study, which was three weeks after tumor implantation.

Impact of anti-PD-L1 treatment on macrophages

As an example of examining the mechanism of action of a drug based on flow cytometry data, we show the analysis of the impact of PD-L1 blockade on the different immune cell subsets in the CT26.wt model. Investigation of the macrophage subsets shows that tumors in vehicle-treated mice contained more M2 than M1 macrophages, helping these tumors uphold an immune-suppressive environment (Figure 3A). During anti-PD-L1 treatment, however, M1 cells proliferated and grew to exceed the amount of M2 cells, which suggests that anti-PD-L1 reprograms the polarization and proliferation of macrophages (Figure 3B). As more immune-activating M1 macrophages are present in the tumors, there is likely increased interference with the immune-suppressive environment, due to cytokine secretion (D.I. Gabrilovich et al. 2012). Furthermore, M1 macrophages

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**Table 1. Frequencies of tumor-infiltrating leukocyte populations.**

(A) Leukocytes comprise the absolute amount of CD45+ cells in the tumors. (B) The individual immune cell subsets are shown relative to each other with font sizes reflecting their frequencies.

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**PD-1 / PD-L1 Pathway**

Programmed cell death ligand 1 (PD-L1) is part of an immune checkpoint required to sustain immune tolerance and induce immune suppression. Many tumor cells express this molecule as a means of immune evasion signalling to programmed cell death protein 1 (PD-1) receptor-expressing immune cells to minimize the immune system’s anti-tumor activity. Immune cells that express PD-1 include activated T cells, macrophages, dendritic cells and natural killer cells.

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clear tumor cells, thereby helping decrease the size of the tumor. The importance of the M2/M1 ratio is demonstrated by the significant correlations to the tumor volume (Figure 3C).

**Impact of anti-PD-L1 treatment on MDSCs**

M-MDSCs were the dominant myeloid subset found in the CT26.wt tumors (Figure 4A). The number of M-MDSCs increased in response to PD-L1 treatment. This subset was more commonly present in small tumors, both with and without anti-PD-L1 treatment (not shown). PMN-MDSCs, on the other hand, were present in larger tumors, suggesting a role to support tumor growth, although only very few PMN-MDSCs were present in CT26.wt tumors. Interestingly, under PD-L1 blockade, this effect disappeared and the PMN-MDSC counts no longer correlated with tumor volume.

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**Table 2. Correlation analysis between tumor volume and immune cell subset frequencies.**

For each tumor, the number of a certain immune cell subset was correlated with the size of the tumor, indicating activating or suppressing roles of the individual immune cell subsets. Negative R²-values (-1 to 0) represent negative correlations, e.g. high numbers of immune cells found mainly in small tumors. Positive R²-values (0 to 1) represent positive correlations, e.g. high numbers of immune cells found mainly in large tumors. Statistically significant values are shown in bolded red font (students t test).
Figure 3. Mechanism of action analysis of anti-PD-L1 antibody based on the macrophages subsets infiltrating CT26.wt tumors.
(A) Anti-PD-L1 treatment increased the proliferation of “good” M1 macrophages and suppressed the proliferation of “bad” M2 macrophages.
(B) The ratio of M2 to M1 macrophages switched from greater than 1 to less than 1 after PD-L1 blockade (students t test).
(C) The ratio of M2 to M1 macrophages negatively correlated with and without anti-PD-L1 treatment, which suggests no impact of the PD-L1 blockade on macrophage function.

* p≤0.05; ** p≤0.01

Impact of anti-PD-L1 treatment on T lymphocytes

Investigation of the T lymphocyte compartment showed a significant increase in the number of all T cell subsets with anti-PD-L1 treatment (Figure 5A). There was a negative correlation between activated CD4+ and CD8+ T cells and tumor growth, suggesting that the effector T cells were actively fighting the tumor (Table 2). However, the same result applied to regulatory T cells, which belong to the immune-suppressive subsets. PD-1 is highly expressed on regulatory T cells. A blockade of this pathway was shown to interfere with their proliferation and to inhibit their immune-suppressive capacities (L.M. Francisco et al. 2009). In accordance with these data, PD-L1 blockade in CT26.wt tumors seems to diminish the suppressive nature of regulatory T cells, as demonstrated by the complete shift in correlation between tumor volume and the ratio of activated CD8+ T cells to regulatory T cells (Figure 5B). Taken together, we can nicely follow the action of anti-PD-L1 treatment using flow cytometric analysis with our three antibody panels to detect macrophages, MDSCs and T cells.

Our findings included repolarization of M2 to M1 macrophages, a loss of function of the PMN-MDSC subset, and proliferation and gain of function of CD8+ effector T cells.
We conclude that PD-L1 blockade works on different immune cell subsets to enable the immune system to effectively kill tumor cells and suppress tumor growth in the CT26.wt model.

Summary
In this white paper we used anti-PD-L1 treatment as an example to demonstrate that proper flow cytometry analysis is a valuable tool for mechanism of action analysis. The characterization and validation of our syngeneic tumor models helps our customers to choose the right model for their drug development approach. We provide flow cytometry data and analysis for all of our models, which will be made available upon request, and we constantly expand the number of available cell lines and immune cell markers.

References
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