Tumor-associated antigen tracking following immunomodulatory radiotherapy

Esben Christensen\(^{1,2}\), Lars Ringgaard\(^{1,2}\), Anja Brus\(^{1,2}\), Hølmtfridur R. Håldorssdottir\(^{1,2}\), Trine B. Engel\(^{1,2}\), Jennifer S. Jørgensen\(^{1,2}\), Andreas Kjær\(^{2}\), Anders E. Hansen\(^{1,2}\), Thomas L. Andresen\(^{1}\)

\(^{1}\)Department of Health Technology, Biotherapeutic Engineering and Drug Targeting, Technical University of Denmark

\(^{2}\)Dept. of Clinical Physiology, Nuclear Medicine & PET and Center for Molecular Imaging, Dept. of Biomedical Sciences, Rigshospitalet and University of Copenhagen

**Background**

Radiation therapy (RT) leads to immunologic cell death of cancer cells where tumor-associated antigens (TAA) is released together with immune stimulatory factors leading to priming of adaptive anti-cancer responses. Immunomodulatory radiotherapy is a promising strategy that combines the strong anti-cancer properties of RT with synergistic immunotherapeutics like Toll-Like-Receptor agonists (TLR-a) that further facilitates immunologic cancer recognition, rejection, and induction of anti-cancer memory. Although presentation of tumor-associated antigen (TAA) is essential for mounting efficient anti-cancer responses, it is often inefficient due to suppressive factors present in the TME, e.g. immune-suppressive subsets such as mononcytic myeloid-suppressor cells (Mo-MDSCs). Overcoming the suppressive milieu is possible by treatment with TLR7 agonists to re-polarize the tumor micro environment (TME) and facilitate antigen presentation leading to an anti-tumor response. In order to evaluate the effects of immunomodulatory radiotherapy, we tracked TAA-uptake in response to treatment using a B16-F10 tumor model and TLR7a-stimulated bone marrow derived dendritic cells (BMDC) as antigen-presenting cells (APC).

**Methods**

The potency of immunomodulatory radiotherapy was determined in efficacy on CT26- and B16-F10-bearing mice (BALB/cUR and C57BL/6JRj, respectively). Mice were inoculated with 2-3x10^5 cancer cells and treatments were initiated on day 3 and 14, respectively (mean tumor size: 1.15 mm\(^3\)). Tumor growth was monitored by means of tumor volume, which was calculated according to the formula: \(V = \frac{d \times D^2}{2}\), where \(d\) is the smallest and \(D\) is the largest diameter of the tumor. Mice were treated with RT (5x2 Gy q1d for CT26 and 1x1.5 Gy for B16-F10) combined with treatments with a intratumoral sustained release depot containing a TLR7a agonist (WL + i.t. NT03).

TAA-uptake was performed using C57BL/6JRj mice inoculated with 5x10^6 B16-F10-mCherry (obtained from Mikael Egeblad, Cold Spring Harbor Laboratory) subcutaneously. Established tumors (mean of 100-130 mm\(^3\)) were treated with 1x15 Gy RT and the TLR7a agonist (WL7a) either i.v. or intratumorally in NT03 gel. Tumor-draining lymph nodes (TDLN) and tumor tissue were excised and processed for flow cytometry. Tumor-draining lymph nodes in tumors were counted on a Muse Cell Analyzer. Both organs were subject to 12-color flow cytometry on a 4-laser BD LSRII Fortessa X-A. All populations were gated as singlet, scatter, viable, and CD45+ and further characterized as: Macrophages (Mφ) and tumor-associated Mφ (TAMs): CD11b+CD11c+CD64+. Patrolling monocytes (pMφ): CD11c+CD103+CD11c+Ly6c+CD64+. Conventional dendritic cells (cDC1): CD11c+CD86+CD8α+Ly6c+. Conventional D2 (cDC2): CD103+CD11c+Ly6c+CD64+CD8α+. Plasmacytoid dendritic cell (pDC): CD123+CD45+CD11c+. B cells: CD19+CD11c+. CD45+CD11c+CD64+CD11b+CD45+CD11c+CD8α+. Red blood cells: CD45−CD11c−CD11b−CD8α−CD45+.

Tumor-draining lymph node cells were stained with antibodies for: CD45+, Mφ (CD11c+Ly6c+), cDC1 (CD11c+CD8α+), pDC (CD123+) and B cells (CD19+). B16-F10 tumor cell expression was measured by Flow cytometry on a Muse Cell Analyzer. The proliferation index was determined by flow cytometry on a Muse Cell Analyzer.

**Antigen Uptake is Associated with CD86 and MHC II**

Antigen uptake was strongly associated with a MHC II+ CD86+ phenotype of relevant myeloid populations in the tumor-draining lymph nodes. The TME was evaluated 7 days after immunomodulatory radiotherapy. Tumor-draining lymph nodes were stained with antibodies for: CD45+, Mφ (CD11c+Ly6c+), cDC1 (CD11c+CD8α+), pDC (CD123+) and B cells (CD19+). B16-F10 tumor cell expression was measured by Flow cytometry on a Muse Cell Analyzer. The proliferation index was determined by flow cytometry on a Muse Cell Analyzer.

**Conclusion**

**Immunomodulatory radiotherapy cures immunogenic cancer models and significantly increases survival in a poorly immunogenic B16-F10 cancer model.**

Left panel: Survival of CT26-bearing mice treated with 5x2 Gy local RT given as an i.t. injection combined with NT03 containing TLR7a in q1d. RT + i.t. NT03 significantly increased survival relative to untreated or NT03 treated groups, with a complete response rate of 100%. Right panel: Survival of B16-F10 wild-type mice with 15 Gy local RT combined with 4x NT03 containing TLR7a given as q1d. Immunomodulatory radiotherapy was initiated on day 5 (mean tumor volume: 11.8 mm\(^3\)).

**Tumor-associated antigen tracking following immunomodulatory radiotherapy**

Figure 1. Concept illustration. mCherry (ph-stable fluorochrome) is expressed in tumor cells and may be taken up by antigen presenting cells (APCs). Thymic mCherry signal represents the cells taking up TAA and whether it is trafficked to TDLN.

**Tumor-Associated Antigen Tracking**

Figure 2. TAA-uptake (mCherry) is highest in pMφ and TAMs in the TME and resident cDC1 in TDLN. TAA-uptake was investigated 1, 4, and 7 days after RT. Left: mCherry+ in malignant populations in tumors. Right: Percentage of mCherry+ positive in malignant populations in tumor-draining lymph nodes.

**Modulation of the TME and TDLN Activation**

Figure 3. TAA mCherry+ uptake is strongly associated with a MHC II+ CD86+ phenotype of relevant myeloid populations in the TME. TAA-uptake was investigated 1, 4, and 7 days after RT. Top row: mCherry+ Mφ in subsets of malignant populations based on 15 Gy local radiotherapy. Single positive or negative refers to MHC II−/CD86− in MHC II+ CD86+ cells. Bottom row: Representative example of the population plotted based on CD64+ and MHC II expression with mCherry overlap.

**Tumor-associated antigen tracking following immunomodulatory radiotherapy**

Figure 4. Immunomodulatory radiotherapy cures tumors in the immunogenic cancer model CT26 and increases survival in the poorly immunogenic B16-F10 cancer model. Left: Survival of CT26-bearing mice treated with 5x2 Gy local RT given as an i.t. injection combined with NT03 containing TLR7a given as q1d. RT + i.t. NT03 significantly increased survival relative to untreated or NT03 treated groups, with a complete response rate of 100%. Right: Survival of B16-F10 wild-type mice with 15 Gy local RT combined with 4x NT03 containing TLR7a given as q1d. Immunomodulatory radiotherapy was initiated on day 5 (mean tumor volume: 11.8 mm\(^3\)).