compared to TNFα. For instance, 35/39 cells (90%) showed IFNγ-mediated up regulation of PD-L1, PD-L2, HLA-ABC and HLA-DR, whereas TNFα induced expression of all immune markers in only 23/39 cells (59%). We also noted variation in the activity of IFNγ and TNFα that reflected melanoma sub-types. For instance, in the 31 cutaneous melanoma cell lines, the relative expression of HLA-ABC and HLA-DR was similar after IFNγ stimulation. In contrast, TNFα preferentially induced HLA-ABC, rather than HLA-DR, in the cutaneous cell lines and HLA-ABC was also preferentially stimulated by both cytokines in the 8 uveal melanoma cell lines. Response of the four immune markers to IFNγ and TNFα was often correlated. This was most evident in the case of PD-L2. We noted strong correlation between baseline, IFNγ- and TNFα-induced PD-L2 levels (Spearman’s rank correlation coefficient: p<0.001, r=0.861 for TNFα- and IFNγ-induced PDL2). Interestingly, we observed only a weak correlation between TNFα- and IFNγ-induced PD-L1 levels (p=0.026, r=0.356), and this may be because neither IFNγ- nor TNFα-induced PD-L1 expression correlated with baseline PD-L1 levels indicating that different transcription factors regulate PD-L1 and PD-L2. Only one cell line (D22) failed to induce immune regulators after IFNγ treatment and this was associated with a missense mutation in the IFNγ receptor 1 (P44R). Thus, although IFNγ signalling was disrupted in immunotherapy resistant melanomas, this is rarely the case in immunotherapy-naïve melanoma cells. Significantly, the D22 cell line responded to TNFα with upregulation of all four markers and we did not observe any cell line that failed to respond to both cytokines.

**Conclusion**

These findings have important implications for immunotherapy, confirming that most melanomas are responsive to IFNγ and/or TNFα.

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**PO-369**

**EFFECTS OF CYCLING HYPOXIA ON THE COMMUNICATION BETWEEN MACROPHAGES AND ENDOTHELIAL CELLS IN PROMOTING TUMOUR GROWTH AND METASTASIS**

V Delprat*, C Tellier, O Feron, C Michiels. *University of Namur, Unité de recherche en biologie cellulaire URBc, Namur, Belgium; 2University of Louvain Medical School UCL, Pôle de pharmacologie et thérapeutique FATH, Bruxelles, Belgium

10.1136/esmoopen-2018-EACR25.880

**Introduction**

Tumours are not only composed of malignant cells. Indeed, blood vessels, composed of endothelial cells, are required for tumour growth. Interestingly, the induction of angiogenesis requires not only endothelial cells but also immune cells. Among them, tumor-associated macrophages (TAMs) make up to 50% of the tumour immune infiltrate. These TAMs are strong angiogenesis inducer and are in part responsible of the tumour inflammation. Two kinds of macrophages are detected in tumours: pro-inflammatory and anti-tumoral M1 macrophages and anti-inflammatory and pro-tumoral M2 macrophages.

Hyoxia is another key feature of tumour microenvironment which induces angiogenesis and enhances tumour inflammation and metastasis. Two types of hypoxia occur in tumour: chronic hypoxia, which impacts cells too far from the blood vessels, and cyclic hypoxia (CyH), which causes intermittent oxygenation of malignant and non-malignant cells.

We have studied the impact of CyH on macrophage polarisation and activity. Secondly, the impact of CyH on the communication between macrophages and endothelial cells was investigated. Then, the impact of this dialogue on tumour growth and metastasis will be studied.

**Material and methods**

M0 macrophages are then polarised into M1 macrophages with LPS and IFNγ (24 hour incubation) or into M2 macrophages with IL-4 and IL-13 (48 hour incubation). After exposing these macrophages to normoxia, chronic hypoxia or CyH, the expression and secretion of pro-inflammatory factors were studied by RT-qPCR and ELISA, respectively. To investigate the communication between macrophages and endothelial cells under CyH, endothelial cells were incubated with CyH-exposed macrophage conditioned-medium and the expression of endothelial cell activation markers was analysed by RT-qPCR.

**Results and discussions**

CyH induced a pro-inflammatory phenotype in M0 macrophages and enhanced the pro-inflammatory phenotype of M1 macrophages. Indeed, an increased expression of TNFα, IL-6 and IL-1β was observed. These effects depended on NF-kB activation since IκK inhibition prevented these effects. The conditioned-media of M0, M1 and M2 macrophages exposed to CyH induced endothelial cell activation as observed by an increased IL-6, IL-8 and ICAM-1 expression.

**Conclusion**

CyH induced a pro-inflammatory phenotype in M0 and M1 macrophages via NF-kB activation. The M0, M1 and M2 macrophages exposed to cycling hypoxia induced endothelial cell activation by a secreted molecule that needs to be identified.

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**PO-370**

**A CO-CULTURE ASSAY SYSTEM USING ENGINEERED ANTI-CD3 TUMOUR CELLS TO ASSESS TUMOUR CELL SENSITIVITY TO CD8+ T CELL KILLING**

N Nelson*, M Lopez-Pelaez, A Palazon, R Wilkinson, S Dowedi, P Smith. AstraZeneca, Bioscience- Oncology- IMED Biotech Unit, Cambridge, UK; MedImmune, Oncology, Cambridge, UK

10.1136/esmoopen-2018-EACR25.881

**Introduction**

Immunotherapy has had a significant impact on the landscape of cancer treatment. However, with only a 20%–30% response rate, identifying novel tumour intrinsic mechanisms of resistance is key to advancing treatment success. Activation of CD8+ T cells, the main cellular mediators of adaptive tumour immunity, requires both T cell receptor (TCR) binding of MHC bound peptides and co-stimulation by antigen presenting cells (APCs). On tumour recognition, antigen-specific T cells become reactivated, leading to tumour cell lysis. Therefore, cytotoxic assays that measure CD8+ T cell responses against tumours, are crucial for understanding therapeutic outcomes.

**Material and methods**

We have developed an in vitro system in which we have engineered tumour cells to express single-chain (scFv) anti-CD3 (clone OKT3) which provides signal 1 to CD8+ T cells, in a co-culture assay. This system allows for understanding the relative contributions of MHC-independent mechanisms to T-cell killing of a panel of tumour cell lines, in parallel. As a model, we have transduced the EGFR mutant non-small cell lung cancer (NSCLC) cell lines PC-9, NCH-H1975 and NCH-H3255 to stably express anti-CD3.

**Results and discussions**

Immune checkpoint blockade yields a clinical response in a subset of NSCLC patients. However, EGFR mutant NSCLC patients show less favourable responses. This assay allows us to investigate how EGFR genotype might
influence tumour cell susceptibility to T cell killing. The pre-activated CD8+ T cells used in the co-culture assay underwent two rounds of anti-CD3/CD28 stimulation to optimise effector function. This resulted in increased CD45RO expression but decreased CD45RA expression and a heightened ability to lyse P815 target cells. Co-cultures of these T cells with tumour cells showed that these cell lines differ in their sensitivities to T cell killing as NCI-H1975 cells appeared to be the most sensitive whilst PC-9 and NCI-H3255 showed similar levels of killing. The flow cytometry based readout also allows for exploring tumour effects on T cell phenotype. Our data showed that all three cell lines significantly induced Granzyme B production in T cells in co-culture, which decreased with increasing E:T ratios and was the lowest with NCI-H1975 cells.

Conclusion Taken together, our results demonstrate that our co-culture system is a robust assay for evaluating T cell-mediated cytotoxicity across cell lines and can potentially be used to investigate the effects of immunomodulatory drugs on T cell killing of different tumour cell lines.

PO-371 ADAM28 DELETION IN MICE INDUCES CD8+ T CELL DECREASE AND IMPACTS THE ONSET OF LUNG METASTASIS

Introduction ADAM28 is highly overexpressed in NSCL cancer. In addition, intrinsic characteristics of this protease argue for considering it as a potential regulator of cellular signalling pathways leading to an inflammatory pulmonary microenvironment and to carcinogenesis. Indeed, ADAM28 bears an active catalytic domain and interacts in a non-proteolytic manner with integrins α4β1 and P-selectin ligands involved in inflammatory cell migration. The aim of this project is to characterise effects of host-ADAM28 on physiological and pathological processes.

Material and methods ADAM28 conditional KO mice have been developed in our laboratory. This unique mouse strain provides a precious tool to investigate ADAM28 implication in lung tumour growth and dissemination. Indeed, mice were intravenously injected with Lewis Lung, 4 T1 carcinoma cells and B16K1 melanoma cells. Since ADAM28 is expressed by thymic epithelial cells and implicated in lymphocyte transendothelial migration, FACS analysis has been performed to study lymphocyte subsets implicated in tumour cytotoxicity or in regulation of immune response.

Results and discussions First analysis showed that there is no spontaneous phenotype for ADAM28 full knockout animals as they are fertile and do not display any abnormality or defect. Surprisingly, an increased metastatic dissemination in lungs has been observed in KO mice compare to WT littermates. Among infiltrating immune cells implicated in anti-tumour response, only CD8 T cells are decreased in metastatic lungs of ADAM28 KO mice. Interestingly, this decrease is already observed in spleen of tumour free-ADAM28 KO mice. These data suggest a potential role of ADAM28 during lymphocyte maturation process, which could further impact antitumoral functions. Moreover, ex vivo analyses of CD8 T cells demonstrate an increase of PD-L1 at cell surface of CD8 T cells isolated from ADAM28 KO mice. PD-L1 interacts with its PD-1 receptor and their interaction induces CD8 apoptosis and exhaustion. Further investigations are required to demonstrate the link between ADAM28 and PD-L1 and their implication in the decrease of CD8 T cell infiltration.

Conclusion The role of tumour cell-derived ADAM28 as a pro-tumour factor is widely described in the literature. However, our results demonstrate an anti-metastatic function of host-derived-ADAM28 suggesting a dual role of ADAM28, depending on the origin of the protease. Based on our results, ADAM28 seems to play a major role in the control of anti-tumour immune response mediated by CD8 T cells.

PO-372 IL-2 AND IL-15 CYTOKINE IN VITRO TREATMENTS INDUCE NKG2D, CD158A AND CD158B RECEPTOR EXPRESSION ON T, NKT-LIKE AND NK CELL LYMPHOCYTE SUBSETS FROM REGIONAL LYMPH NODES OF MELANOMA PATIENTS

1A Vuletić*, 1J Jovanić, 2Z Milovanović, 3I Spurnić, 4V Jurišić, 5G Konjović, 1Institute of oncology and radiology of Serbia, Department of experimental oncology, Belgrade, Serbia; 2Institute of oncology and radiology of Serbia, Department of pathohistology and citology, Belgrade, Serbia; 3Institute of oncology and radiology of Serbia, Department of surgery, Belgrade, Serbia; 4University of Kragujevac, Faculty of Medicine, Kragujevac, Serbia

Introduction Regional lymph nodes (LN)s are important immunological barriers in spreading of malignant tumours but also represent the most frequent early metastatic site in melanoma. Considering that cytokine therapy has shown substantial toxicity, there is a growing need for further in vitro testing of these agents to enlighten aspects of their regional application in malignancies. The aim of this study was to investigate the effect interleukin (IL)–2 and IL-15, cytokines with similar immune-enhancing effects, on the expression of activating (NKG2D) and inhibitory (CD158a,CD158b) receptors on CD8+ T, NKT-like and NK cell lymphocyte subsets from regional LN of melanoma patients.

Material and methods Mononuclear cells (MNC) were purified from regional LN of 35 melanoma patients (clinical stages II-IV) and in vitro cultured for 7 days in cell culture medium RPMI1640 (CM) alone, CM with 200 IU/ml rhIL-2 and CM with 25 ng/ml IL-15. Expression of NKG2D, CD158A and CD158B receptors on lymphocytes, CD8+ T, NKT-like and NK cells was estimated by flow cytometry. Statistical significance between the values obtained with cytokine treatments was evaluated by Wilcoxon signed rank test. NK cell cytotoxicity was evaluated by standard 51Cr release assay.

Results and discussions Our results show that IL-2 and IL-15 in vitro treatments significantly increase expression of activating NKG2D receptor on lymphocytes and their CD8+ T, NKT-like and NK cell subsets. Regarding investigated inhibitory receptors, the significant increase was obtained for CD158a only after IL-15 treatment on lymphocytes and their CD8+ T and NKT-like subsets. However, the expression of CD158b receptor significantly increased after IL-2 cytokine treatment on lymphocytes, CD8+ T and NK cells, and after IL-15 treatment on all investigated lymphocyte subsets.

Both cytokines augmented NK cell antitumor cytotoxicity that positively correlated (p<0.01, Spearman signed rank test) with the expression of NKG2D2 activating receptor on NK