**Programmed cell death-1 receptor mediated regulation of Tbet+NK1.1-Innate Lymphoid Cells within the Tumor Microenvironment**

**Materials and Methods**

**Cell lines**

B16F10 melanoma and MC38 adenocarcinoma colon cancer were kindly provided by Dr Arunakumar Gangaplara, NCI, NIH. Tumor cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS (Labtech), sodium pyruvate (1mM; Sigma) and penicillin-streptomycin (100 units/ml penicillin; 100g/ml streptomycin; GibcoTM) and maintained at 37oC with 5% CO2. C8161 human melanoma cell line and MET1 human squamous cell carcinoma (SCC) cell line (Ximbio) were kindly donated by Professor Penny Lovat, Newcastle University. C8161 cells were cultured with DMEM media supplemented with 10% FBS (Labtech), sodium pyruvate (1mM; Sigma) and penicillin-streptomycin (100 units/ml penicillin; 100g/ml streptomycin; GibcoTM). MET1 cells were cultured with DMEM/F12 media (4:1) supplemented with 10% FBS (Labtech), penicillin streptomycin (100units/ml penicillin; 100g/ml streptomycin), hydrocortisone (0.4g/ml; Sigma), cholera toxin (8.5ng/ml; Sigma), tri-iodo-L-threonine (20pM; Sigma), Adenine (180M; Sigma), Insulin (5g/ml; Sigma), epithelial growth factor (EGF; 2pg/ml; Sigma) and transferrin (5g/ml; Sigma).

**Tumor Models**

*Subcutaneous tumor models of B16F10 and MC38*

WT or B6.*Pdcd1-/-*, B6.*Pdcd1-/-*TbetZsGreen, B6.TbetZsGreen, B6.*Rag2*-/- mice were inoculated with 2 x 105 B16F10 melanoma cells or MC38 cells via subcutaneous injection into the flank as previously reported 56. Tumor volume was recorded daily from day 5 post inoculation. Tumor volume was calculated by the following equation: Tumor volume = /6 x 0.5 x length x width2 as described previously 57. In certain experiments, mice were treated with anti-PD-1 (200g/mouse; clone: RPMI-40); anti-NK1.1 (200g/mouse; clone:PK136) or isotype control (IgG2a, 200g/mouse, clone: 2A3; IgG1a, 200g/mouse; C1.18.24 respectively) via intraperitoneal injection at day 7, 9 and 11 unless otherwise indicated. Mice were euthanized at day 12, unless otherwise stated for tumor infiltrating lymphocyte (TIL) immunobiology assays. In some experiments, animals were injected with BODIPY (200g/mouse) or vehicle before harvest. None of these experiments included exogenous IL-33 administration.

*Metastatic melanoma*

WT or *B6.TbetZsGreen or B6.Pdcd1-/ - TbetZsGreen* mice were inoculated with either 0.5 or 2 x 105 B16F10 melanoma cells in 200l of PBS via intravenous tail-vein injection. Mice were monitored for clinical signs of weight loss and euthanized at >25% weight loss. In certain experiments mice were treated with anti-PD-1 (200g/mouse; clone: RPMI-40) or isotype control (IgG2a, 200g/mouse; clone: 2A3) at day 7, 9, 11. Tissue was harvested at day 12 and analyzed for the presence of ILCs unless stated otherwise. None of these experiments included exogenous IL-33 administration.

*AOM-DSS induced colorectal cancer*

The AOM-DSS model was set up as previously reported 58. Briefly, mice were treated with one dose of Azoxymethane (AOM; 12mg/kg) at day 1 and then 3% dextran sodium sulphate (DSS) was added as drinking water from day 3 for a week. Mice were allowed to recover for 3 weeks and then a 2nd cycle of DSS was started. After the 3rd cycle, animals were euthanized and immunobiology studied. None of these experiments included exogenous IL-33 administration.

**ILC isolation from tumor and normal tissue**

*TIL isolation*

ILCs from the tumor tissue were isolated as previously described 59. Briefly, tumor tissue was incubated at 37oC for 30minutes in FBS free DMEM media containing liberase TLTM (0.25mg/ml; Roche) and DNAse I (0.5mg/ml; Roche). Single-cell suspensions were prepared by mechanically disrupting tissue through a 100m nylon cell strainer into FBS. Lymphocytes were isolated using lymphocyte separation media (LSM; Promocell) and washed twice with complete media (DMEM supplemented with 10% FBS, glutamine (2mM), non-essential amino acids (0.1mM), sodium pyruvate (1mM), 2-mercaptoethanol (50M) and penicillin and streptomycin (100 units/ml penicillin; 100g/ml streptomycin; GibcoTM) to remove traces of LSM. Cells were then analyzed by flow cytometry or stimulated to induce cytokine production. Ethical approval for experiments conducted on human tissue was provided by the South Central Hampshire B NRES Committee (reference number 07/H0504/187). Fresh tissue samples of cSCC and non-lesional skin were obtained from patients during surgery at the Dermatology Department, University Hospital Southampton NHS Foundation Trust. For lymphocyte isolation, samples of tumor and separately, non-lesional skin, were finely disaggregated with scalpels, incubated at 37oC for 1.5 hours in RPMI media containing collagenase I-A (1mg/ml; Sigma-Aldrich) and DNAse I (10µg/ml; Sigma-Aldrich). The resulting suspension was then passed through a 70µm cell strainer and centrifuged over an Optiprep (Sigma-Aldrich) density gradient. Lymphocytes were then extracted and washed with PBS before use in experiments. In certain experiments, TILs were incubated for 60 mins with 2NBDG (200g/ml) or PBS and then ILCs analyzed by flow cytometry.

*ILC isolation from spleen*

Single cell-suspension of splenocytes was generated by mechanically disrupting tissue through a 40m filter into complete media. Cells were incubated with red blood cell (RBC) lysis buffer (Biolegend) for 3 minutes at room temperature and were then washed in complete media once before analysis by flow cytometry.

*ILC isolation from tumor draining lymph nodes*

Tumor draining lymph nodes (TDLN) were isolated from the inguinal draining lymph nodes. Single cell-suspension was generated by mechanically disrupting TDLNs through a 40m filter into complete media. Cells were then washed and analyzed by flow cytometry.

*ILC isolation from lungs*

Lungs were perfused with PBS *in situ* via the pulmonary artery prior to isolation. Tissue was incubated at 37oC for 15 minutes in FBS free DMEM media containing LiberaseTM TL (0.25mg/ml; Roche) and DNAse I (0.5mg/ml; Roche). Single cell suspension was generated by mechanically disrupting tissues through a 100µm filter into FBS followed by a Percoll gradient centrifugation (40% Percoll, GE Healthcare; FBS free media containing 0.5mg/ml DNase, Roche). Cells were then washed with complete media and analyzed by flow cytometry.

*ILC isolation from small intestine*

Small intestine tissue was harvested into complete media. Fecal matter was removed, and tissue was washed in buffer (PBS containing 5% FBS, Hepes (1mM; Sigma), 50M 2-mercaptoethanol (50M; Sigma) and penicillin and streptomycin (100 units/ml penicillin; 100g/ml streptomycin; GibcoTM)). This was followed by a wash with PBS to remove traces of FBS. Tissue was incubated at 37oC for 30 minutes in FBS free DMEM media containing LiberaseTM TL (0.25mg/ml; Roche) and DNAse I (0.5mg/ml; Roche). Single cell suspension was generated by mechanically disrupting tissues through a 100µm filter into FBS. This was followed by percoll gradient centrifugation (40% Percoll (GE Healthcare), containing 0.5mg/ml DNase (Roche)). Cells were washed with complete media and analyzed by flow cytometry.

*ILC isolation from skin*

Mouse normal dorsal skin was harvested, finely chopped and incubated for 2 hours in FBS free DMEM media containing LiberaseTM TL (0.25mg/ml; Roche) and DNAse I (0.5mg/ml; Roche) at 37oC. Single-cell suspensions were prepared by mechanically crushing tissue slurry through a 100m nylon mesh into FBS. Cells were then subsequently filtered through 70- and 40-m nylon filters. Cells were then analyzed via flow cytometry.

**Antibodies**

All the antibodies used to characterize murine and human ILCs were purchased from BioLegend or eBioscience unless otherwise stated. For analysis of murine cell surface markers, the following antibodies were used: Lineage consisted of CD3 (clone: 1452C11), CD5 (clone: 53-7.3 ), CD8 (clone: 53-6.7), CD11b (clone: M1170), CD11c (clone: N418), CD19 (clone: 1D3/CD19), CD49b (clone: HM2), Ter119 (clone: TER-119 ), Gr1 (clone: RB8-9C5), F4/80 (clone:BM8), NK1.1 (clone:PK136), B220 (clone:RA3-8B2). ILCs were stained for CD45 (clone:30-F11), CD90.2 (clone: 30-H12), CD127 (clone: A7R34), CD25 (clone: PC61), KLRG1 (clone:2F1/KLRG1), NKp46 (clone:29A1.4), CD49a (clone: HMα1), PD-1 (clone: RPMI-30), PDL-1 (clone:10F.9G2), PDL-2 (clone: Ty25). ST2 (clone:DJ8) was purchased from MD Bioproducts. For ILC analysis in human peripheral blood mononuclear cells (PBMC), the following fluorochrome-conjugated antibodies were used: Lineage consisting of CD3 (clone: OKT3), CD5 (clone: L17F12 ), CD14 (clone: M5E2), CD16 (HIB19), CD19 (HIB19), CD20 (2H7), CD56 (clone HCD56), CD11b (clone:ICRF44), CD11c (clone: 3.9) and TCR/ (clone: IP26), CD45 (clone:H130), CD127 (clone:A019D5), CD161 (clone:HP-3G10), c-kit (CD117;clone:104D2), CRTH2 (clone:BM16), PD-1 (EH12.2H7) and NKp44 (clone:P44-8). For analysis of murine cytokine production, the following fluorochrome-conjugated antibodies were used: IL-5 (clone: TRFK5), IL-10 (clone: JES5-16ES), TNF (clone: MP6-XT22), IFN (clone: XMG1.1), RORt (clone: B2D), EOMES (clone: Dan11mag), IL-17 (clone: eBio17B7), IL-22 (clone: IL22JOP), IL-13 (clone: eBio13A) and Ki67 (clone: So1A15). Human PBMCs were stained with the following intracellular fluorochrome-conjugated antibodies; Tbet (clone: 4B10), IFN (clone: 4S.B3), IL-17 (clone: BL168), IL-5 (clone: TRFK5), TNF (clone: Mab11), RORγt (clone: B2D) and IL-13 (clone: JES10-5A2).

**Flow cytometry**

Single cell suspensions were generated from indicated organs and stained with Live/Dead fixable dead cell stain kit as per manufacturer’s instructions (Invitrogen). For murine ILC analysis, cells were incubated with biotin labeled lineage cocktail (CD3+, CD5+, CD8+, CD11b+, CD11c+, CD19+, CD49b+, Ter119+, F4/80+, B220+, NK1.1+ and Gr1+) followed by streptavidin. Cells were then stained with a combination of markers including CD45, CD90.2, CD127, CD25, KLRG1, NKp46, PD-1, PDL-1, PDL-2 and ST2. For analysis of NK and myeloid immune subsets, TILs were stained with NK1.1, CD49a, CD49b, F4/80, Gr1, CD11b and CD11c. Cells were then fixed and permeabilized for intracellular markers as follows: Tbet, ROR, Ki67 and EOMES. In order to measure murine intracellular cytokine (IC) production, TILs were stimulated with cytokine stimulation cocktail (Invitrogen; Thermo Fisher Scientific) for 4 hours at 37oC. Cells were then fixed and permeabilized (Fixation/Permeabilization kit; BD Bioscience). ILCs were then stained with: IL-5, IL-13, IL-17, IL-22, IFN- and TNF-.

Human PBMCs and TILs were washed with PBS prior to staining. 1 x 106 cells were stained with Live/Dead fixable dead stain kit as per manufactures instructions (Invitrogen). Cells were then incubated with cell surface antibodies: Lineage cocktail BV510 or FITC (CD3+, CD5+, CD11b+, CD11c+, CD14+, CD16+, CD19+, CD20+, CD56+ and TCR/+), CD45, CD127, CD161, CRTH2, c-Kit (CD117), NKp44 and PD-1. Cells were then fixed and permeabilized (Fixation/Permeabilization kit; BD Bioscience) and stained for intracellular transcription factors as follows: Tbet and RORγt.

ILCs were defined by the following gating strategies: Murine ILCs were defined as Lin- Thy1+; ILC2s were defined as CD127+CD25+KLRG1+/-ST2+/-; NCR+ILC3s were defined as RORt+NKp46+ and NCR- ILC3s were defined as RORt+NKp46-, murine ILC subset regulated by PD-1 was defined as Lin- Thy1+ NK1.1-Tbet+NKp46-Eomes-.  Human ILCs were defined as follows: Lin-CD45+ CD127+. ILC1s were further defined as CRTH2-CD117- ; ILC2s were defined as CRTH2+CD117-; ILC3s were defined as CRTH2-CD117+. Cells were analyzed using BD LSR Fortessa X20 with FACs DIVA software (BD Bioscience) and analysis was performed with FCS Express (De Novo) or FlowJo 10.1 software (Tree Star).

**Rhapsody Single Cell Sequencing**

TILs were isolated as previously described from tumors and then cells were stained with lineage markers (lineage gate included CD3+, CD4+, CD5+, CD8+, CD11b+, CD11c+, CD19+, CD49b+, Ter119+, F4/80+, B220+ and Gr1+), Thy1, and Abseq antibodies and sample Tags. Abseq antibody-oligos used were as follows: CD25, CD103, CD119, CD37, CD223, CD272, CD273, CD274, CD278, CD279, IL17Rb, IL23R, IL33R, CD335, CD3 and NK1.1. Cells were incubated for 20 minutes at 4C and then washed three times with Miltenyi buffer. TILs were then stained with DAPI and flow sorted for Lineage-Thy1+ population. Samples were then pooled and loaded on to rhapsody cartridges and then experiment were performed as per manufacturer’s instructions. Data analysis was performed using the SeqGeq software.

**Transwell assays**

Splenocytes were RBC lysed and then plated at a concentration of 0.5 x 106 cells per ml. Transwell inserts (0.4 micron; ThermoFisher Scientific) were seeded with B16F10 melanoma cells at a 1:1 ratio with splenocytes (unless otherwise stated) and were incubated for indicated time points at 37oC prior to flow cytometry analysis. For proliferation assays, transwell inserts were removed after 6 hours. For human experiments, PBMCs were acquired from healthy donors and were cultured at a concentration of 0.5 x 106 per ml. Transwell inserts were seeded with either C8161 human melanoma cell line or human cSCC cell line at a 1:1 ratio with PBMCs. For human experiments, transwell inserts were removed after 16 hours. Plates were incubated at 37oC for indicated time points and were then analyzed by flow cytometry.

***In-vitro* Proliferation Assays**

For cell trace violet experiments, murine splenocytes isolated from B6.TbetZsGreen mice were stained in PBS with Cell Trace Violet (Invitrogen) as per manufactures instructions. Cells were cultured with IL-2 (40ng/ml), IL-7 (40ng/ml), αPD-1 (20g/ml; clone: RMP1-14) or Isotype IgG2a (20g/ml; clone: 2A3) as indicated for 5 days in cell culture media (DMEM supplemented with 10% FBS, glutamine (2mM), non-essential amino acids (0.1mM), sodium pyruvate (1mM), 2-mercaptoethanol (50M), penicillin and streptomycin (100 U/M)). Cytokines were replenished on day 2 and day 4. Proliferation was measured on day 5 by flow cytometry. For human proliferation assays, human PBMCs acquired from healthy donors were stained in PBS with Cell Trace Violet (Invitrogen) as per manufacturer’s instructions. Cells were cultured with IL-2 (40ng/ml), IL-7 (40ng/ml), αPD-1 (20g/ml; clone:EH12.2H7) or Isotype IgG1 (20g/ml; clone: MG1-45) as indicated for 7 days in cell culture media (RPMI supplemented with 10% FBS, glutamine (2mM), non-essential amino acids (0.1mM), sodium pyruvate (1mM), 2-mercaptoethanol (50M), penicillin and streptomycin ((100 units/ml penicillin; 100g/ml streptomycin; GibcoTM)). Cytokines were replenished on day 2 and day 4. Proliferation was measured on day 7 by flow cytometry.

**Lactate Assays**

WT or *Pdcd1-/ -*splenocytes were incubated with IL-2 (100 ng/ml) plus IL-7 (100 ng/ml) alone or in combination with lactic acid (20 mM) for 24 hrs and then PD-1 expression was measured by flow cytometry. For human studies, 1x106 PBMCs were incubated with IL-2 (100 ng/ml) plus IL-7 (100 ng/ml) alone or in combination with lactic acid (20 mM) for 24 hrs and then PD-1 expression was measured by flow cytometry within the LineagenegCD45+CD127+CRTh2-CD117-Tbet+ subset. B16F10 tumor cells were expanded and then supernatant was tested for lactic acid production as per the manufacturer’s instructions (Abcam). Briefly, 2x106 cells were seeded in 24 well plates and then supernatant harvested after 4hrs or 24 hrs. The amount of lactic acid was determined using a lactic acid fluorometry kit.

**Phospho-P70S6Kinase Measurement**

Tumors were resected when they reached >600mm3 and then TILs were isolated. TILs were stimulated with IL-2 (80ng/ml) and IL-7 (40ng/ml) for 15 minutes. TILs were washed once with PBS and then stained for phospho-p70S6Kinase antibody and then analyzed by flow cytometry. In some experiments, TILs were enriched using CD90.2 microbeads as per manufacturer’s instructions and then cultured for 3 days with IL-2 plus IL-7 alone or in combination with isotype (20g/ml) or anti-PD-1 antibody (20g/ml). At day 3 post cultures, TILs were washed with complete media once and then restimulated with IL-2 and IL-7 for 15mins. Following stimulation, phosphorylation of p70S6Kinase was measured.

**Statistical Analysis**

Statistical analysis was performed with GraphPad Prism using an unpaired T test for groups of two and a ONE-WAY ANOVA for multiple groups. Results are expressed as mean± standard error of the mean (SEM) and P-values ≤0.05 were considered significant. Survival curve analysis was performed using a Kaplan-Meier survival curve and a log rank test.