Healthy Volunteers

Density Gradient Medium

EDTA-Blood

PBMC

Undesired Cells remain in Coumns

T cells in Eluate

Purified CD8 T cells (n = 10)

Purified CD4 T cells (n = 10)

Unstimulated CD8 T cells (n = 10)

Stimulated CD8 T cells (n = 10)

Unstimulated CD4 T cells (n = 10)

Stimulated CD4 T cells (n = 10)

Cell Lysis for RNA and Protein Extraction

Confirm Expression
of human proteins such as
CD334, EREG etc in Tregs
(*which are known
to harbour
STAT3-enhancing pTyr-SNV*)
RT-QPCR
Immunoblots
Flow Cytometry

1.7.2.2-a.

Confirm STAT3 function
in human Tregs

Flow Cytometry Assays

1.7.2.2-b.

**Cancer Patients' Tissue Archive
(Biobank)**

PBMC

Genotyping
Flow Cytometry Assays
RT-QPCR
Immunoblots

1.7.2.2-c & d.
1.7.3.2-a.

Immunohistocheimistry
Immunofluorescence

RT-QPCR
Immunoblots

1.7.2.2-c & d.
1.7.3.2-a.

Disease-Free Survial
Treatment Response

1.7.2.2-c & d.
1.7.3.2-a.

Online Supplementary Figure-1. Flow chart showing the methodology described in Project Description Section 1.7.2.2 to study melanoma-associated SeSNVs using healthy PBMCs, archived tumour materials, and patient treatment response data.
To determine the expression of receptors known to harbour SeSNVs in human T cells, we will sort Tregs, CD8, and CD4 T cells from the blood gender-matched consenting human volunteers, with a minimum cohort size of n=10. The purified immune cells from each individual will be split into several portions. One portion will be used for transcript analysis via RT-QPCR, while the other will be used for protein analysis through immunoblotting. The data obtained from RT-QPCR and immunoblotting will be validated and complemented by conducting intracellular and surface staining experiments with flow cytometry and immunofluorescence-compatible antibodies.

To assess the preferential expression of STAT3 by blood-borne immune cells, we will evaluate the survival and proliferation potential of monocytes, macrophages, Tregs, CD4, and CD8 T cells within human peripheral blood mononuclear cells (PBMCs) using flow cytometry-based assays following the pharmacological inhibition of STAT3. The specific cell types that are preferentially depleted as a result of STAT3 inhibition are expected to be highly reliant on the STAT3 signalling pathway.

Using archived melanoma tumour tissue sections from University BioBanks (UMG & UKT), and the associated patient metadata, we will deploy RT-QPCR, immunoblots, immunohistochemistry, and immunofluorescence to quantify the relative number of tumour-infiltrating Tregs, CD4, and CD8 T cells. To determine the correlation between CD8 T cells in the TME and melanoma progression, two methods will be employed: IHC, IF, RT-QPCR, and IB analyses will be performed to deduce a reliable and reproducible quantitative estimation of CD8 T cell infiltration per unit of tumour area and adjacent normal tissues. Furthermore, RT-QPCR, immunoblot, and double immunofluorescence will be performed to detect and quantify the expression of cytotoxic molecules, cytokines, and interleukins. To determine the association of SeSNVs with the anti-CD8 T-cell response, we will perform statistical correlation analyses between genotyping data and the quantification of CD8 T cells in the tumour and blood compartments.