**ABSTRACT**

**BACKGROUND:** Only two K⁺ channels are known to be expressed by human T-cells. Activated effector T cells express high levels of K,1,3; activated naive and central memory T-cell subsets express high levels of K,3.1. Overexpression of K,1,3 in murine T-cells increases K⁺ efflux, improves effector function (IFN-γ production) and promotes anti-tumor activity. Inhibition of K,3.1 suppresses murine T-cell proliferation and cytokine production. Thus, activation of K⁺ channels enhances TIL growth and could promote T-cell effector function resulting in mediating tumor regression.

**RESULTS:** In human PBMCs and TIL K,3.1 expression was relatively low with upregulation observed within 24 h following stimulation with anti-CD3 and anti-CD28. TIL propagated in the Rapid Expansion Protocol (REP) had a 1.42-fold greater expansion (p=0.002) in the presence of SKA-31 and significant increases in the CD8⁺CD28⁺ (p=0.04), CD8⁺CD27⁺ (p=0.04), and CD8⁺CD27⁺CD28⁺ subpopulations (p=0.002), consistent with a less differentiated phenotype. Significantly increased K,3.1 was found in pre-REP TIL samples (n=8) as compared to normal donor PBMCs (n=6) in both CD4 and CD8 subpopulations (p=0.0016). Addition of K,3.1 agonist SKA-31 in pre-REP TIL notably heightened CD25 and CCR7 expression.

**CONCLUSION:** We demonstrate that SKA-31 treatment enhances CCR7 expression associated with memory cells, promotes TIL expansion, and attenuates T-cell differentiation. Targeting the K,3.1 channel is a novel strategy to expand and sustain less differentiated TILs and may improve the clinical application of adoptive T-cell therapy with TIL.

**METHOD**

**LION’s TIL Expansion Process.**

The tumor is excised from the patient and transported to the GMP Manufacturing Facility. Upon arrival the tumor is fragmented and placed in G-Rex flasks with IL-2 for TIL expansion (pre-REP expansion).

**Figure 1.** pre-REP TIL were propagated with REP using irradiated PBMCs, anti-CD3 (30 ng/mL), IL-2 (6000 IU/mL) alone or with K,3.1 agonist SKA-31 treatment. The impact of SKA-31 treatment was assessed as a function of fold expansion and phenotypic analysis.

**Figure 2.** K,3.1 is widely expressed by all T-cell subsets in normal donor PBMCs T-cell subset is defined using CD45RA and CCR7, namely naive, central memory (TCM), effector memory (TEMRA), and effector memory RA⁺ (TEMRA) cells. Normal donor PBMCs were stained with anti-CD3, anti-CD4, anti-CD8, anti-K,3.1, anti-CD45RA, and anti-CCR7 and analyzed by flow cytometry (n=6). Percentage of K,3.1 expression is demonstrated in each T-cell subset of CD3⁺CD4⁺ (A, B) and CD3⁺CD8⁺ (C, D). No statistical difference in K,3.1 expression in each T-cell subset is found using student’s unpaired T test. p values <0.05 are considered statistically significant.

**Figure 3.** K,3.1 expression is up-regulated following T-cell activation. Normal donor PBMCs were activated with anti-CD3 (1000 ng/mL) and anti-CD28 (500 ng/mL) (n=6). Pseudocolor plots demonstrate the percentage of K,3.1 in CD3⁺CD4⁺ subset (A) and CD3⁺CD8⁺ subset (C) on day 0 and day 3 following TCR activation. Kinetic expression of K,3.1 within 11 day time course is demonstrated in CD3⁺CD4⁺ (B) and CD3⁺CD8⁺ subsets (D).

**Figure 4.** Heightened expression of K,3.1 in pre-REP TIL. K,3.1 expression was assessed by flow cytometry in normal donor PBMCs (n=6) and pre-REP TIL (n=8). Pseudocolor plots and dotplots represent the percentage of K,3.1 expression demonstrated in CD3⁺CD4⁺ (A, B) and CD3⁺CD8⁺ subsets (C, D) of both normal donor PBMCs and TIL. p values represent the difference between normal PBMCs and pre-REP-TIL using student’s unpaired T test. p values <0.05 are considered statistically significant.

**Figure 5.** SKA-31 enhances TIL expansion with more sustained expression of CD27 and CD28 suggesting a less differentiated phenotype. Pre-REP TIL were propagated with Rapid Expansion Protocol (REP) using irradiated PBMCs, anti-CD3 (30 ng/mL), IL-2 (6000 IU/mL) alone or with SKA-31 for 14 days. Comparison of TIL expansion between no treatment and SKA-31 is demonstrated as fold expansion (n=10). (A) CD3⁺CD8⁺CD28⁺ subset. (B) CD3⁺CD8⁺CD28⁺ subset. (C) CD3⁺CD8⁺CD27⁺CD28⁺ subset (D) were assessed in post-REP TIL in both no treatment and SKA-31 treatment groups (n=10). p values represent the difference between no treatment and SKA-31 using student’s T test. p values <0.05 are considered statistically significant.

**Figure 6.** SKA-31 Enhances Expression of CD25 and CCR7. Pre-REP TIL were grown with either IL-2 (6000 IU/mL) alone or with SKA-31 (n=14). CD25 and CCR7 expressions were assessed by flow cytometry in CD4⁺ (A, B) and CD8⁺ (C, D). p values represent the difference between no treatment and SKA-31 using student’s paired T test. p values <0.05 are considered as statistically significant.

**SUMMARY**

- K,3.1 was expressed by all peripheral blood T-cell subsets including naive, central memory (TCM), effector memory (TEMRA), and effector memory RA⁺ (TEMRA) cells.
- Profound up-regulation of K,3.1 was identified within 24 hours following T-cell activation.
- TIL have significantly higher level of K,3.1 as compared to normal T-cells in the peripheral blood which suggests that TIL are activated T-lymphocytes.
- Activation of the K,3.1 channel with the K,3.1 agonist (SKA-31) promotes TIL expansion; this is potentially due to increased CD25 expression.
- SKA-31 helps sustain CD27 and CD28 expression during TIL expansion.
- Heightened CD25 and CCR7 expression was observed in pre-REP TIL grown with IL-2 in combination with SKA-31.
- Activation of the K⁺ channel could be a novel strategy to promote TIL expansion and sustain a less differentiated phenotype, promoting long term engraftment.

**References**