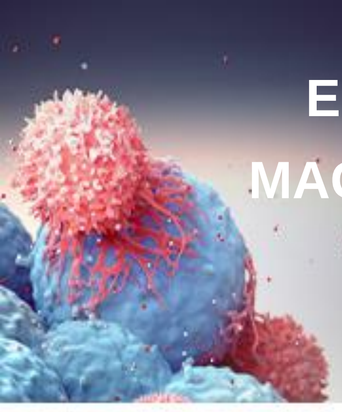


Enhanced anti-tumor activity and T-cell fitness of 2nd-generation MAGE-A1 TCR T-cells incorporating distinct CD8 co-receptor designs

Paul Najm, Josephine Kemna, Friederike Knipping, Marleen Van Loenen, Panagiota A. Sotiropoulou, Mikhail Steklov

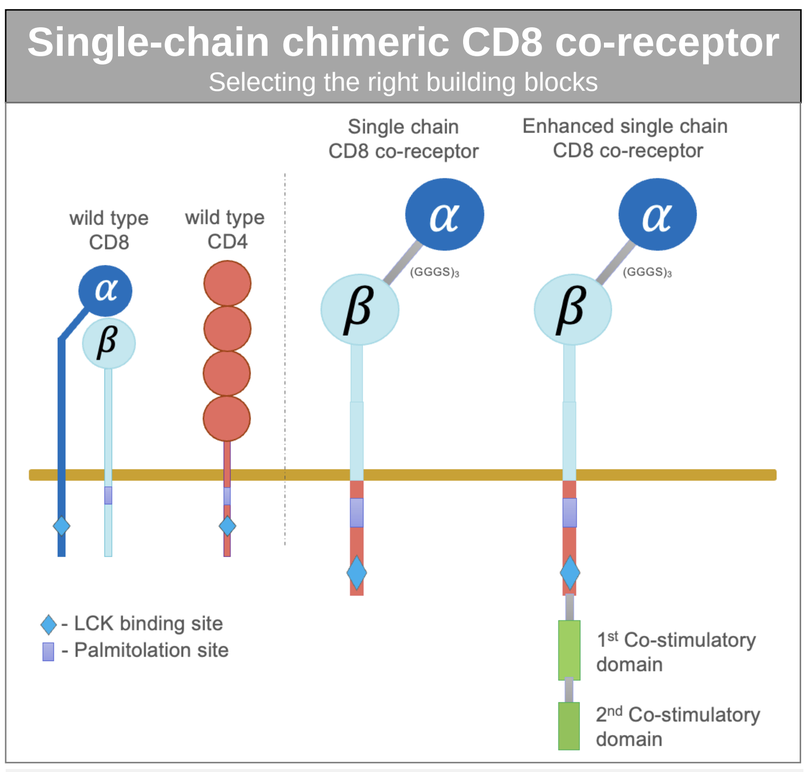
T-knife Therapeutics Inc., San Francisco, USA

Background

Next-generation T cell receptor-engineered T cell therapy (TCR-T) technologies include redirection of CD4+ T cells to peptide-MHC class I complexes through incorporation of a CD8 co-receptor (CD8 CoR). Early clinical and extensive preclinical validation demonstrated that CD8CoRs can enhance anti-tumor activity and improve T cell fitness[1]. Currently known CD8 CoR approaches include (i) a wild-type (wt) CD8 CoR, comprised of a CD8-alpha (CD8a) and a CD8-beta (CD8b) chain, and (ii) a single CD8a chain[2]. In studies comparing head-to-head these approaches the wt-CD8 CoR displayed superior preclinical activity compared to single CD8a chain, providing CD4+ T cells with cytotoxic activity while maintaining immune modulating functions[3], but construct size and incorporation of multiple elements remain a challenge. We therefore designed chimeric CD8 CoRs, combining building blocks from the CD8a and CD8b chains and the CD4 co-receptor. The smaller construct allowed for the addition of two tumor necrosis factor (TNF) receptor-associated factors (TRAFs) binding motifs, derived from TNF receptor family proteins, combining engagement of CD4+ T cells with the enhanced function of both CD4+ and CD8+ T cells. We then engineered different CD8 CoRs with a MAGE-A1-targeting TCR, currently under clinical development (NCT05430555), and selected the design that enabled the highest anti-tumor response.

Methods

CD4+ and CD8+ T cells were transduced with a MAGE-A1 TCR alone or together with different CD8 CoRs. Constructs were analyzed for transgene expression levels and phenotype by flow cytometry. Real-time cytotoxicity upon long-term or repeated stimulation using different effector-to-target ratios (E:T ratios) was measured by IncuCyte Zoom S3, and cytokine secretion profile was determined by LEGENDplex ™ (BioLegend).



Multiple chimeric single-chain CD8 CoRs were created by combining structural and functional domains from the CD4 and CD8 CoRs. The selected chimeric single-chain CD8 CoR (scCD8 CoR) encompasses all functional elements of the wild-type CD8 CoR in a smaller single-chain construct: both alpha and beta heads, respective CD8 stalk and TM, in combination with the CD4 intracellular domain. Enhanced chimeric single-chain CD8 CoR (escCD8 CoR) includes two additional co-stimulatory domains separated by a short linker.

1. A single-chain chimeric co-receptor endowing CD4 T cells cytotoxic activity via an HLA class I-restricted TCR was designed by combining functional domains from the wild-type CD4 and CD8 co-receptors

As a first step, we generated the functional backbone of a chimeric single-chain chimeric CD8 CoR (scV19) enabling effective engagement of CD4 T cells, without negatively impacting CD8 T cell function.

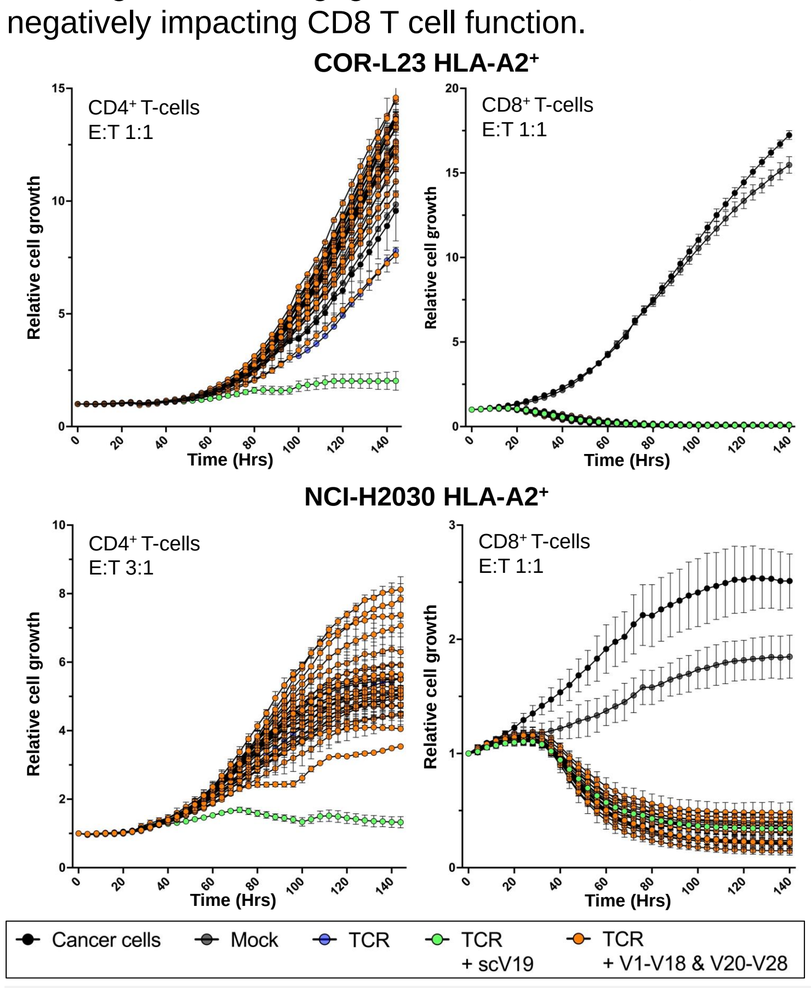


Fig.1: Isolated CD4 and CD8 T cells transduced with MAGE-A1 TCR alone, or in combination with different single-chain chimeric versions of CD8 CoR were co-cultured with COR-L23 cells at effector-to-target (E:T) ratio 1:1, and NCI-H2030 cells at E:T ratio 3:1 (CD4) or 1:1 (CD8). Both cell lines naturally express MAGE-A1 and overexpress HLA-A*02:01. Data are shown as mean ±SD of 3 technical replicates.

2. Incorporation of scV19 chimeric CD8 CoR and a MAGE-A1-targeting, HLA class I-restricted TCR bestows functional activity to CD4 T cells without negative impact on CD8 T cells

Next, we performed thorough comparison of the initial chimeric design (scV19) with the wt-CD8 CoR.

scV19 displayed similar cytotoxicity to that of wt-CD8 CoR, but showed a slight trend for lower cytokine secretion, indicating requirement for further optimization.

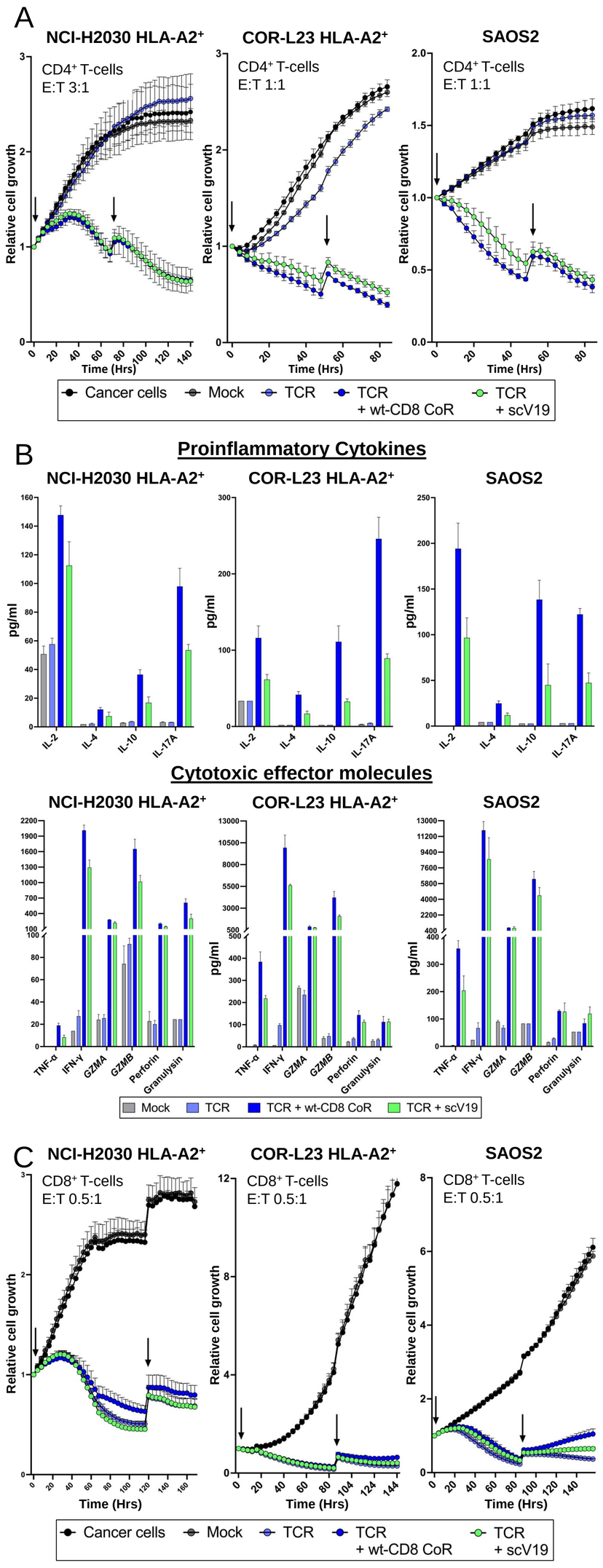


Fig.2: Isolated CD4 (A,B) and CD8 (C) T cells transduced with MAGE-A1 TCR alone, or in combination with wt-CD8 CoR or scV19 were co-cultured with HLA-A*02:01-overexpressing NCI-H2030 or COR-L23 cells, or with SAOS2 cells, all naturally expressing MAGE-A1, at the indicated E:T ratios. Arrows indicate addition of fresh tumor cells. Cytokine secretion was determined 72 hours post co-culture with the indicated cell lines. Data are shown as mean ±SD of 3 technical replicates.

3. Optimized version of scV19 chimeric CD8 CoR displays improved functional activity, similar or higher than the wt-CD8 CoR

We incorporated three point mutations to scV19 to increase its expression levels. This optimized construct (scCD8 CoR) resulted in enhanced activity, leading to higher cytotoxicity and similar secretion of cytotoxic effector molecules compared to wt-CD8 CoR when cultured with cancer cells at low E:T ratio (E:T = 0.25:1).

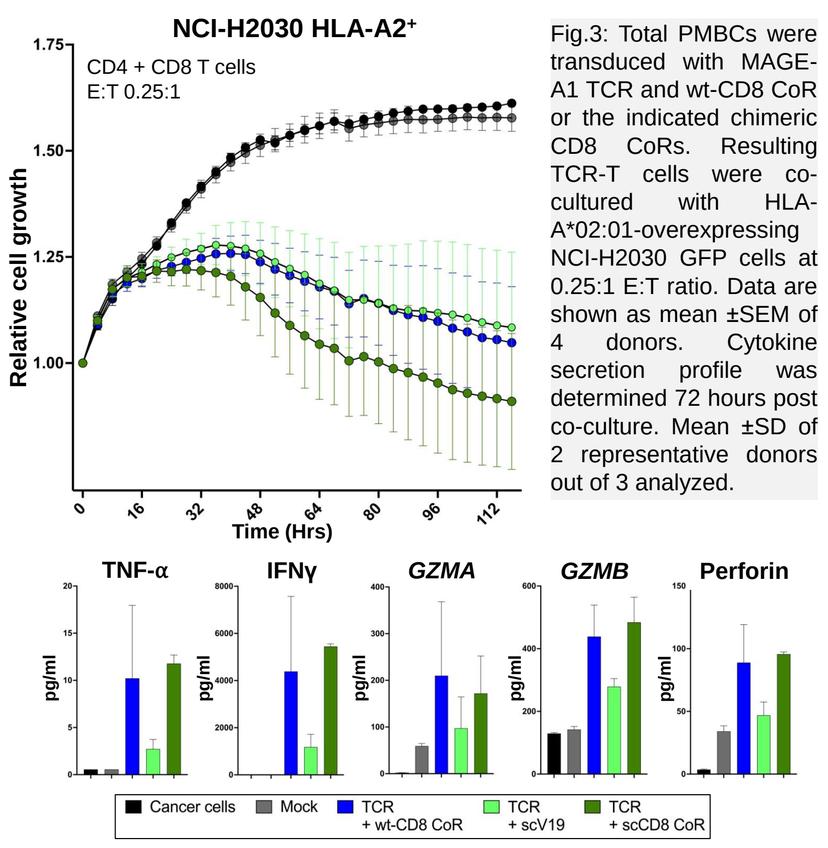


Fig.3: Total PMBCs were transduced with MAGE-A1 TCR and wt-CD8 CoR or the indicated chimeric CD8 CoRs. Resulting TCR-T cells were co-cultured with HLA-A*02:01-overexpressing NCI-H2030 GFP cells at 0.25:1 E:T ratio. Data are shown as mean ±SEM of 4 donors. Cytokine secretion profile was determined 72 hours post co-culture. Mean ±SD of 2 representative donors out of 3 analyzed.

4. Addition of TRAF6 and TRAF1,2,3 binding motifs to the single-chain co-receptor leads to an enhanced single-chain CD8 CoR (escCD8 CoR) that mediates improved T cell phenotype and boosts cytotoxic activity and cytokine secretion compared to wt-CD8 CoR

The small size of the single-chain CD8 co-receptor enables it to serve as a scaffold, adding combinations of costimulatory domains to counterbalance the lack of co-stimulatory molecules on cancer cells.

Surprisingly, commonly used co-stimulatory domains, such as CD28 and 4-1BB, did not improve CD4 T cell cytotoxicity. In sharp contrast, the addition of TRAF-binding motifs from TNF receptor family members enabled sustained cytotoxic activity upon repeated antigen stimulation (data not shown). The single-chain co-receptor incorporating TRAF6 and TRAF1,2,3 binding motifs (enhanced single-chain CD8 CoR; escCD8 CoR) was selected for further investigation.

In depth characterization of TCR-T cells bearing the escCD8 CoR and the wt-CD8 CoR demonstrated that the escCD8 CoR leads to earlier memory phenotype at harvest, improved long-term cytotoxicity at very low E:T ratios and enhanced secretion of effector molecules, indicating the potential to mediate longer and deeper responses.

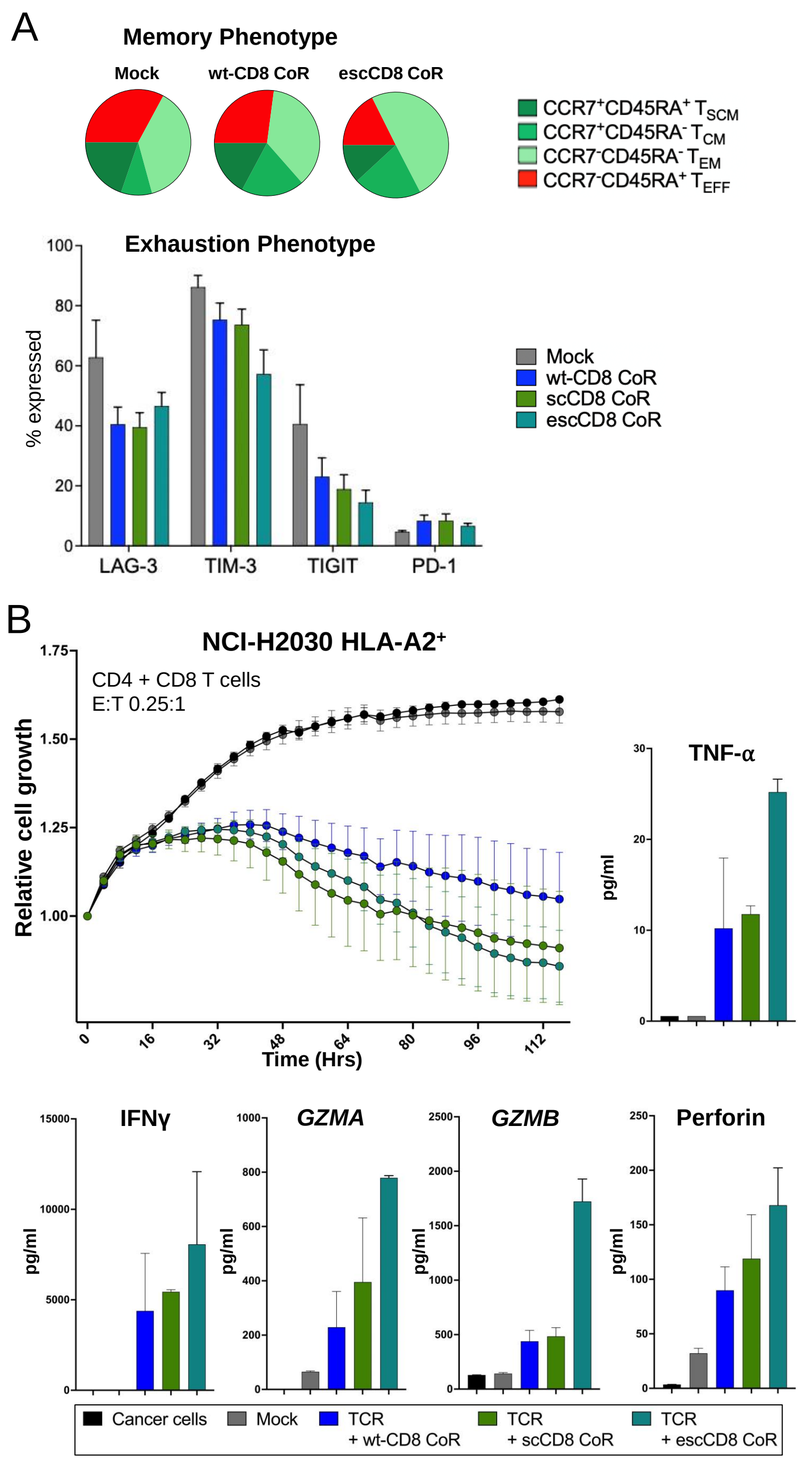


Fig.4: (A) Phenotype of transduced T cells was determined by flow cytometry. Memory phenotype was evaluated based on CCR7 and CD45RA expression, and exhaustion profile was assessed by LAG-3, TIM-3, TIGIT and PD-1 levels. Data are shown as mean ±SEM of 4 donors. (B) TCR-T cells bearing the indicated CD8 CoRs were co-cultured with HLA-A*02:01-overexpressing NCI-H2030 GFP cells at E:T ratio 0.25:1. Cytokine secretion was determined 72 hours post co-culture. Mean ±SD of 2 representative donors out of 3 analyzed.

Conclusions

- Combining functional domains from CD4 and CD8 co-receptors, with or without TRAF binding motifs, we created two single-chain chimeric CD8 co-receptors with essential properties for TCR-T therapy.
- The scCD8 CoR, without additional co-stimulation, provides CD4 T cells with improved functional activity compared to the wt-CD8 CoR, with reduced payload and number of 2A cleavage sequences.
- The escCD8 CoR, incorporating TRAF6 and TRAF 1,2,3 binding motifs, results in earlier memory phenotype in both CD4 and CD8 T cells, and enhanced long-term cytotoxicity and pro-inflammatory cytokine secretion upon co-culture with cancer cells in very low E:T ratios.
- Incorporation of the chimeric CD8 CoRs to TCR-T cells provides the potential for enhanced persistence and efficacy, and consequently a more profound antitumor effect.

References

1. Rath, Jan A., et al. (2020). *Science advances*, 6(27), eaaz7809.
2. Anderson, V. E., et al. (2023). *Journal of Immunotherapy* (Hagerstown, Md.: 1997), 46(4), 132.
3. Bajwa, G., et al. (2021). *Journal for ImmunoTherapy of Cancer* I (Suppl 2), A173-A173

www.t-knife.com