

# Functional TILs from human renal cell carcinoma

## Generation of functional tumor-infiltrating lymphocytes from human renal cell carcinoma using the gentleMACS™ Dissociator and the Tumor Dissociation Kit

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### Background

Adoptive transfer of tumor-infiltrating lymphocytes (TILs) is a promising approach for the treatment of patients with advanced malignant melanoma.<sup>1</sup> As both malignant melanoma and renal cell carcinoma (RCC) are sensitive to IFN- $\gamma$ - and IL-2-based immunotherapy, cellular therapy involving TILs might also be appropriate for the treatment of RCC patients.

However, clinical trials aiming to elucidate the clinical efficacy of adoptive transfer of RCC TILs showed variable results.<sup>2,3</sup> This was mostly due to unsuccessful production of TILs from RCC and the variation in TIL phenotype. It is therefore highly desirable to establish reliable methods enabling consistent generation of tumor-specific TILs from RCC.

We here describe a combined mechanical/enzymatic process for the dissociation of renal tumors, involving the gentleMACS™ Dissociator and the Tumor Dissociation Kit from Miltenyi Biotec, enabling the generation of high numbers of functional TILs with well-preserved cell surface epitopes.

### Materials and methods

#### Tumor samples

Tumor samples were obtained from patients with renal carcinoma from three hospitals in the South Manchester region. All patients gave their informed consent. Sample collection was approved by the local ethics committee (LREC 09/H1003/75). Alternatively, samples were acquired from the Manchester Cancer Research Centre Biobank.<sup>4</sup>

#### Tumor dissociation

Renal tumor samples were dissociated into single cells either by an established overnight digestion method or a semi-automated combined mechanical/enzymatic process using the gentleMACS Dissociator and the Tumor Dissociation Kit, human (Miltenyi Biotec). The overnight digestion of small tumor pieces (2–3 mm) was carried out using an enzyme mix including DNase (3,000 U/mL), collagenase (10 mg/mL), and hyaluronidase (10 mg/mL), at room temperature under continuous rotation.<sup>5</sup>

Tumor dissociation with the gentleMACS Dissociator was done according to the manufacturer's instructions. The tumor tissue was cut into pieces of 2–3 mm in size and transferred into C Tubes (Miltenyi Biotec) containing a mix of Enzymes H, R, and A (Tumor Dissociation Kit, human; Miltenyi Biotec). Mechanical dissociation was accomplished by performing three consecutive automated steps on the gentleMACS Dissociator (h\_tumor\_01, h\_tumor\_02, and h\_tumor\_03). To allow for enzymatic digestion, the C Tube was rotated continuously for 30 min at 37 °C, after the first and second mechanical dissociation step.<sup>4</sup>

#### Expansion of TILs

Following tumor dissociation, the samples were passed through 100  $\mu$ m filters and the resulting single-cell suspensions were cultured in RPMI 1640, supplemented with 10% FCS, 1% glutamine, 100 IU/mL penicillin, 100  $\mu$ g/mL streptomycin, 25 mM HEPES, and 50  $\mu$ M  $\beta$ -mercaptoethanol, in 24-well plates at a cell density of  $0.5 \times 10^6$  cells/mL. The culture also contained 3,000 IU/mL recombinant IL-2. To achieve substantial expansion, the cells were cultured in the presence of CD3 and CD28 antibodies bound to large particles (bead:T cell ratio = 1:1). On day 7,

the IL-2 concentration was reduced to 1,000 IU/mL and the particles were removed. Subsequently, cells were expanded by dilution to maintain a density of  $10^6$  cells/mL. Cultures were expanded until day 15.<sup>4</sup>

### Flow cytometry

Uncultured TILs from tumor samples dissociated either by overnight digestion or the gentleMACS Dissociator were labeled with CD3, CD4, CD8, and CD45 antibodies and analyzed by flow cytometry.<sup>4</sup>

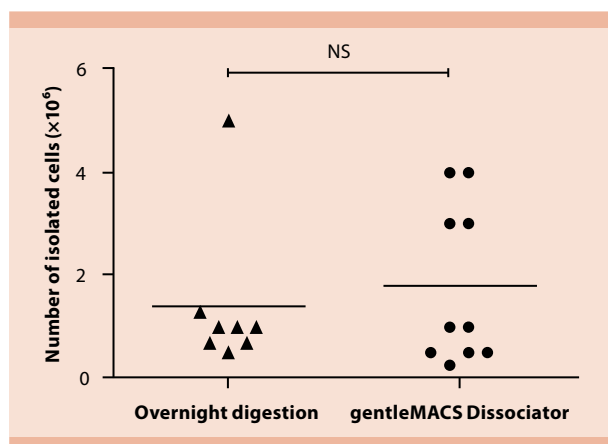
### Coculture of TILs and autologous tumor cells, IFN- $\gamma$ assay

To determine their functional capacity, expanded TILs were analyzed for IFN- $\gamma$  secretion in response to stimulation with tumor cells. To this end, the TILs were cocultured with autologous tumor cells at a ratio of 1:1 ( $10^5$  cells per well). The tumor cells were derived from the initially dissociated tumor samples, which were cryopreserved in 10% DMSO and 90% FCS until use. The coculture was maintained for 24 h at 37 °C. Subsequently, the culture supernatants were analyzed for IFN- $\gamma$  using an ELISA.<sup>4</sup>

## Results

### Combination of mechanical and enzymatic tumor tissue dissociation on the gentleMACS™ Dissociator yields high numbers of TILs

We compared the two methods for the dissociation of renal tumor tissue to obtain TILs: the novel, combined mechanical/enzymatic, automated 3-hour process involving the gentleMACS™ Dissociator versus an established overnight digestion protocol<sup>5</sup>. Both methods resulted in similarly high numbers of TILs (fig. 1).

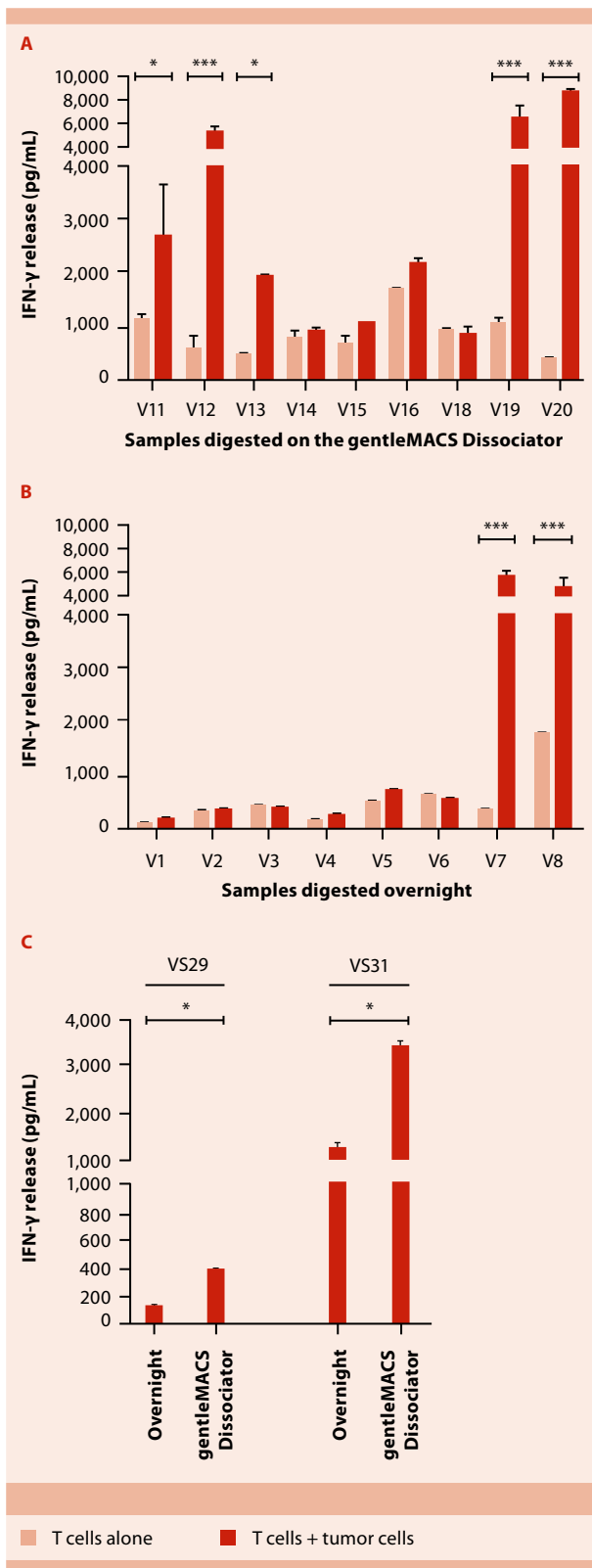


**Figure 1: Tumor dissociation using overnight digestion or a mechanical/enzymatic process on the gentleMACS Dissociator.** Tumor samples were dissociated by overnight digestion ( $n = 8$ ) or the gentleMACS Dissociator ( $n = 9$ ) as indicated. The number of TILs was determined using a Neubauer hemocytometer chamber. There was no significant difference between the two sample groups (NS: not significant at  $p > 0.05$ , Mann-Whitney test). Data were adapted from reference 4.

TILs generated by both methods could also be expanded at similar rates with no significant difference. In a 15-day culture period, TILs generated by the gentleMACS Dissociator expanded  $19.1 \pm 4.4$ -fold (range 45–2.5; median 15.6), and TILs obtained through the overnight digestion  $30.4 \pm 7.4$ -fold (range 68–2; median 24.5).

### TILs generated by the gentleMACS™ Dissociator showed more pronounced IFN- $\gamma$ secretion in cocultures with autologous tumor cells

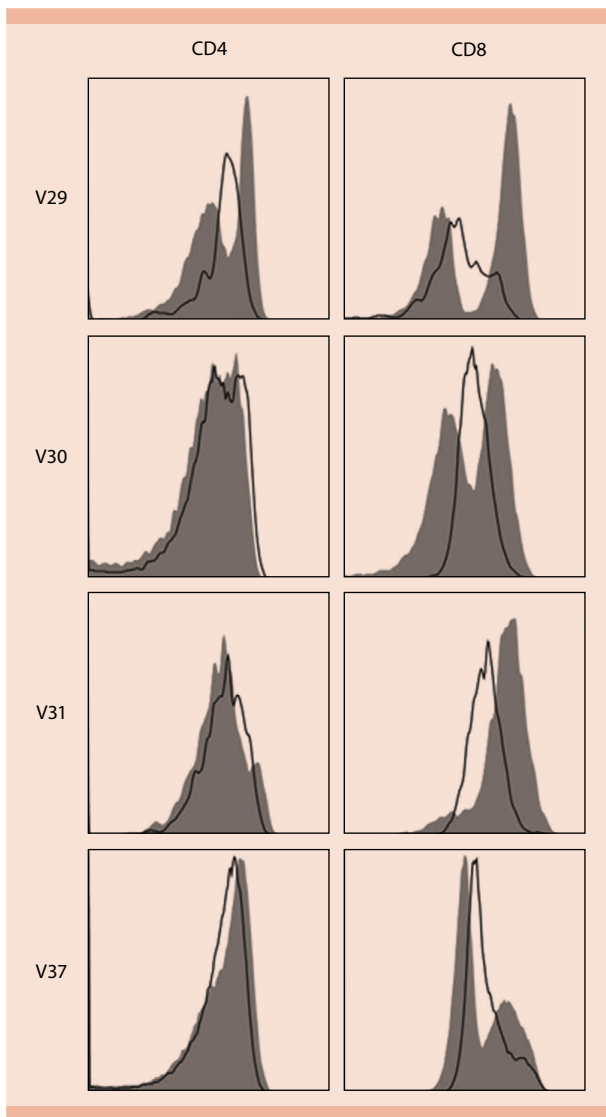
The secretion of IFN- $\gamma$  is one of the hallmark functions of antigen-specific T cells encountering the respective antigen. We therefore analyzed expanded TILs, initially generated by the two methods, for their capacity to secrete IFN- $\gamma$  in a coculture with autologous tumor cells. Five out of nine expanded TIL cultures (56%), initially generated using the gentleMACS™ Dissociator, showed an increase in IFN- $\gamma$  secretion in the presence of autologous tumor cells compared to TILs alone (fig. 2A). In contrast, only two out of eight TIL cultures (25%) derived from the overnight digestion showed a significant increase in IFN- $\gamma$  secretion (fig. 2B). These data suggest that TILs generated with the gentleMACS Dissociator have a higher capacity to secrete IFN- $\gamma$  compared to TILs generated with the overnight digestion. As this effect might be simply due to differences in the tumor biopsies, we also tested both methods with identical samples in parallel. Two tumor samples from different donors were available that were large enough for the preparation of two identically sized pieces. The two pieces were then processed with the two tumor dissociation methods side by side. Figure 2C confirms the previous result indicating that TILs generated with the novel mechanical/enzymatic process show a more pronounced IFN- $\gamma$  secretion than TILs from samples dissociated by overnight digestion.



**Figure 2: Functional analysis of expanded TILs.** TILs were generated by combined mechanical/enzymatic dissociation of tumor tissue on the gentleMACS Dissociator (A) or overnight tissue digestion (B). TILs were then either cultured alone or cocultured with autologous tumor cells at a ratio of 1:1 for 24 h. (C) Tumor samples from two donors were processed with both tumor dissociation methods in parallel to allow for direct comparison. Cell culture supernatants were analyzed for IFN- $\gamma$  by ELISA (A–C). Data are means $\pm$ SEM of three experimental replicates (\* $p < 0.05$ ; \*\*\* $p < 0.001$ ; two-way ANOVA with Bonferroni post-test correction). Data were adapted from reference 4.

### TILs generated with the gentleMACS™ Dissociator showed higher expression levels of cell surface markers

Anti-tumor functionality of TILs depends on the expression of certain surface markers. However, tissue dissociation involving enzymatic treatment has the inherent risk of proteolytically degrading cell surface proteins. CD4 and CD8 are important coreceptors involved in antigen recognition by T cells. We therefore analyzed the expression of CD4 and CD8 on TIL samples generated with the two methods in parallel. To this end, four TIL samples from different donors were labeled with CD3, CD4, CD8, and CD45 antibodies after tumor dissociation, without culturing, for flow cytometry. Figure 3 shows the analysis of CD3<sup>+</sup>CD45<sup>+</sup> T cells. All four samples generated with the gentleMACS™ Dissociator showed a higher CD8 expression compared to samples from the overnight digestion. One out of four samples processed with the gentleMACS Dissociator also showed a higher CD4 expression. These data suggest that the combined mechanical/enzymatic process is more gentle than the overnight digestion and results in a better preservation of surface epitopes on the TILs, compared to the overnight digestion.



**Figure 3: Expression of surface markers, CD4 and CD8, on non-cultured TILs, prepared by overnight digestion or the gentleMACS Dissociator.** TILs were generated from tumor samples from four different donors. After tumor dissociation, TIL samples were labeled with CD3, CD4, CD8, and CD45 antibodies, and analyzed by flow cytometry. The histograms show the analysis of CD3<sup>+</sup>CD45<sup>+</sup> T cells. Empty lines and filled lines indicate TILs generated by overnight digestion or the combined mechanical/enzymatic process on the gentleMACS Dissociator, respectively. Data were adapted from reference 4.

## Conclusion

- Combined mechanical/enzymatic dissociation of renal tumors using the gentleMACS Dissociator and the Tumor Dissociation Kit preserves cell surface epitopes more effectively than the established overnight digestion protocol.
- Dissociation of renal tumor cells with the gentleMACS Dissociator results in high numbers of TILs. The numbers are equal to the established overnight digestion. Expansion rates of TILs generated by both methods are similar.
- TILs generated with the gentleMACS Dissociator show a more pronounced IFN- $\gamma$  secretion compared to TILs obtained by overnight digestion.

## References

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