



Miltenyi Biotec

Generation of tumor-infiltrating lymphocytes

Tumor immunology

gentleMACS™ Dissociation of melanoma tumors for the generation of tumor-infiltrating lymphocyte cultures for adoptive cell therapy

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Introduction

TIL (tumor-infiltrating lymphocyte) therapy can cause regression of bulky melanoma lesions and mediate durable responses in refractory cancer patients. TIL therapy consists of using a patient's own tumor-reactive lymphocytes, which have been activated and expanded to large numbers in the laboratory. The primary method for initiation of TIL has traditionally been the overnight digestion of fresh tumor tissues in a triple enzyme medium. This method is open, uses non-FDA-approved enzymes (hyaluronidase) and is operator-dependent. The gentleMACS™ Dissociator from Miltenyi Biotec is a mechanical tissue dissociation device, which potentially simplifies and standardizes tissue dissociation for the generation of TIL. The gentleMACS Dissociator works by disrupting the extracellular matrix and cell adhesion components without harming the integrity of the cell membrane. This is achieved by a combination of varying enzyme mixes, mechanical forces, incubation periods, and temperatures. The automation of all mechanical steps, using the gentleMACS Dissociator, has led to reproducible results with reduced overall processing times. It is a closed system, utilizes single-use, disposable supplies, has rapid processing times, and can be standardized.

We examined the gentleMACS Dissociator for generation of TIL cultures for research and clinical use. Our early experiments using the instrument explored multiple

variables. Qualitative and quantitative aspects of tissue processing were optimized, including cell yield and viability, flexibility for diverse clinical samples, technical simplicity, and regulatory compliance. Some variables considered included gentleMACS Program settings, inclusion of enzymes, the composition of enzymes, incubation times, and sample size.

This analysis resulted in a standard operating procedure (SOP) and the identification of a minimum number of variables to optimize.

We next focused on optimizing an SOP for the gentleMACS Dissociator and comparing it with the current clinical SOP to process fresh tumor for the generation of TIL. Multiple head-to-head comparisons were performed to evaluate: i) the initial dissociation product for the total cell yield, lymphocyte/tumor cell ratio, and percentage of viable cells; ii) the resulting TIL cultures for initial expansion, phenotype, and cell function; iii) clinical-scale expansion and clinical efficacy.

Material and methods

Dissociation of fresh tumor tissues

All samples collected and used were derived from patients who signed an informed consent approved by the institutional review board of the NIH. All patients receiving treatment on this study were treated as part of a clinical protocol.¹

On the day of tumor resection, the specimen was received in the laboratory immediately following surgery. The specimen was bathed in sterile saline in a sterile container. Viable tumor tissue was dissected away from the majority of non-viable tissue and healthy (non-tumor) tissue in a laminar flow hood using a scalpel and sterile forceps. Research samples were collected during this dissection, including procurement of tissue for confirmation of diagnosis by pathology. Other research specimens included flash frozen tissue for RNA isolation, tissue cryopreserved in OCT for histopathology, an FNA sample for cytopathology, and separate samples for tumor tissue culture lines. The

tumor sample was weighed and kept in a 50-mL centrifuge tube containing a small amount of sterile HBSS if processed the same day, or in 10-mL sterile Complete Medium (CM) at 4 °C if it was to be processed after overnight storage. The complete medium for culturing TIL was prepared by supplementing RPMI 1640 with 10% human serum (heat-inactivated at 56 °C for 30 minutes), and with final concentrations of penicillin G (100 units/mL), streptomycin (100 µg/mL), gentamicin (50 µg/mL), Hepes (25 mM), and 2-mercaptoethanol (5.5×10^{-5} M). Selected antibiotics for relevant allergies were omitted.

The tissue specimen was placed on a sterile cutting surface (cutting board or an open Petri dish). Using sterile scalpel and forceps the specimen was cut into small (3–5 mm) fragments. Cut fragments were transferred into a gentleMACS C Tube. Enzyme medium (EM) was added to the tube (5 mL for 0.2–2 g of tissue and 10 mL for 2–5 g) and the tube was securely closed by turning the cap until a slight click was heard. EM was used to help disperse tumor cells from the surgical specimen during the short incubation periods between the gentleMACS Program runs. The enzyme-containing medium, RPMI 1640, did not contain serum. It had the following added components (final concentrations): penicillin G (100 units/mL), streptomycin (100 µg/mL), gentamicin (50 µg/mL), Fungizone® (1.25 µg/

mL), Collagenase (1 mg/mL) and Pulmozyme® (~30 units/mL).

The enzyme stocks were dry powders stored at –20 °C. The C Tubes were installed vertically and cap-side down into the gentleMACS Sleeve. Proper installation ensured that the C Tubes were held in position against rotational and axial forces. Pre-defined programs are provided by the internal gentleMACS Dissociator memory. The programs vary in intensity, so an appropriate program was selected depending on the texture of the tissue to be processed. Softer tissues required a more gentle rotation (lower speed) and harder tissues required a more vigorous, longer rotation. For the human tumor tissues received by this lab, we have found the following series of programs and incubations to be optimal:

h_tumor_01.01 program

30 minutes incubation at 37 °C

h_tumor_02.01 program

30 minutes incubation at 37 °C

h_tumor_03.01 program

After the first program had run (**h_tumor_01.01**), the C Tube was removed from the gentleMACS Dissociator and placed in a 37 °C incubator for 30 minutes. Subsequently, the C Tube was installed into the gentleMACS Dissociator for its second program run (**h_tumor_02.01**). After another

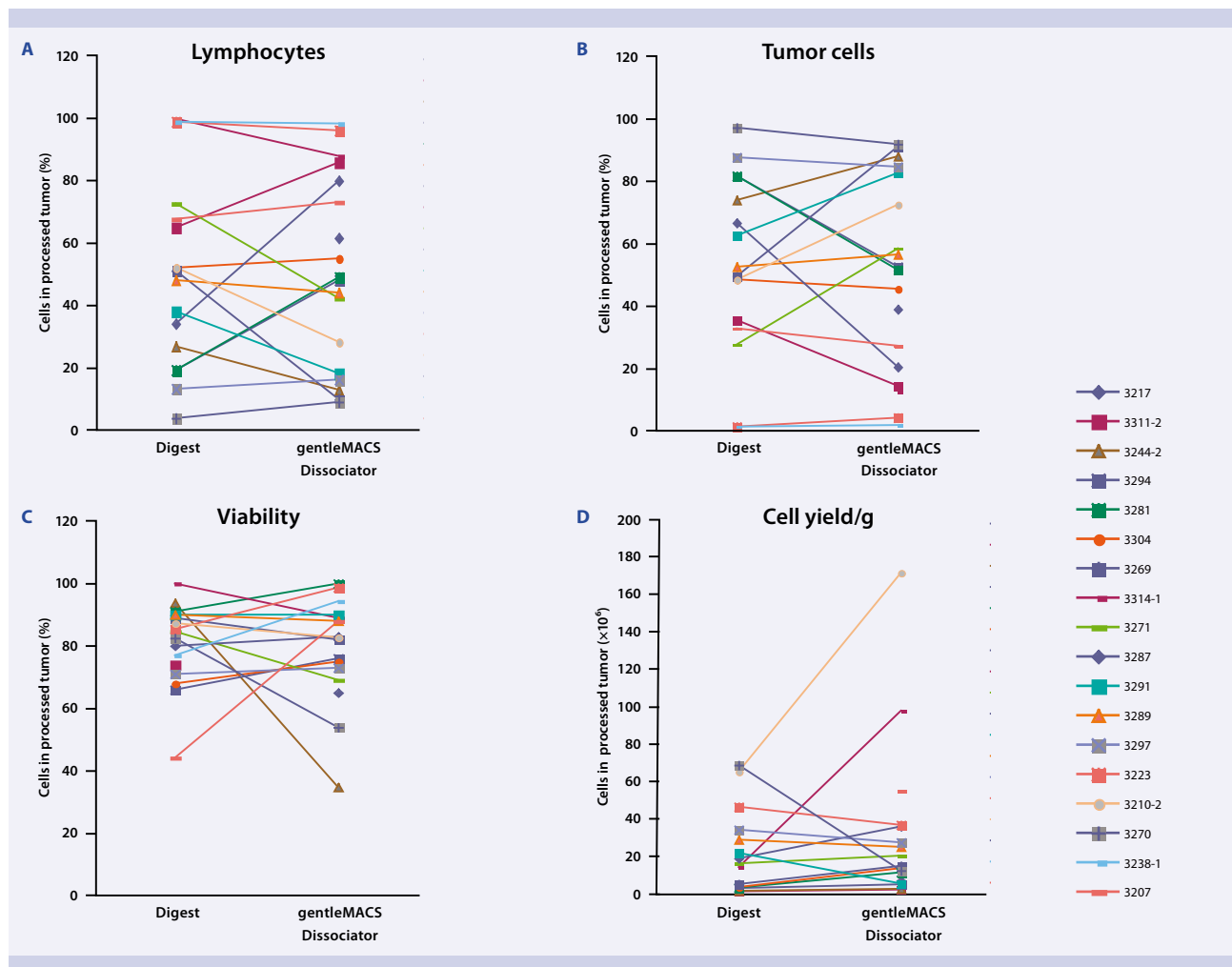


Figure 1 Comparison of the gentleMACS Procedure with the overnight digestion procedure with regard to cell numbers, viability, and cell yield. For comparison, samples from 18 patients were processed using the gentleMACS Dissociator or overnight digestion. Relative cell numbers of TIL (A) and tumor cells (B) as well as viability (C) and cell yield (D) were determined as described in Materials and methods.

30 minutes incubation step the third program run (**h_tumor_03.01**) was performed. After the final program run, the tissue appeared mostly dissociated. If significant chunks of tissue were macroscopically visible, one or two additional gentleMACS Dissociation runs were applied to the tissue, with or without one additional 30-minute incubation step at 37 °C.

The dissociated tissue from the C Tube was filtered through an autoclaved wire mesh placed in an autoclaved funnel on top of a 250 mL centrifuge tube. The wire mesh was rinsed with sterile HBSS and the tube was filled with additional sterile HBSS. The dissociated tissue was then washed one time by centrifuging 10 minutes at 1,500 rpm.

The supernatant was aspirated and the pellet resuspended in a known volume of HBSS.

Processing dissociated single-cell tissue samples and generation of TIL cultures

The resulting single-cell suspension was counted using a hemocytometer with trypan blue exclusion to analyze cell yield. Total numbers of lymphocytes, tumor cells, and red blood cells (RBC), as well as overall cell viability were calculated. If the cell suspension had extensive cellular debris, or the RBC:nucleated cell ratio exceeded 6:1, or the overall viability was lower than 50%, the sample was further processed over a Ficoll® gradient. If the cell suspension did not need to be cleaned up with a Ficoll gradient, then the final wash was completed as follows: the cells were centrifuged (10 minutes, 1,500 rpm), and resuspended in CM. Cultures were set up in 24-well tissue culture plates at 0.5×10^6 total live cells/mL (2 mL/well) in CM containing 10% human AB serum and IL-2 (6000 IU/mL). Remaining cells were frozen at 10^7 live cells/vial. TIL were cultured as previously described.²

TIL cultures were monitored daily for expansion, which was determined by microscopically counting viable cells using trypan blue exclusion and a hemocytometer.

Functional analysis of TIL

Selected TIL were evaluated for tumor recognition by interferon- γ (IFN- γ) release assay as previously described.²

Clinical-scale expansion and therapeutic administration

Successfully generated TIL were used to treat patients in ongoing surgery branch clinical protocols. These TIL were rapidly expanded and administered to metastatic melanoma patients.¹

Statistical comparisons

Head-to-head comparisons were tested with a paired t-test. Populations were compared using a student's 2-sided t-test assuming unequal variance. Fisher's exact test was used to evaluate responses vs. source of TIL. All responses were considered significant for $P < 0.05$. Patients treated between May 2009 and May 2010 (inclusive) under Surgery Branch, NCI Protocols #07-C-0176, were included in this analysis.

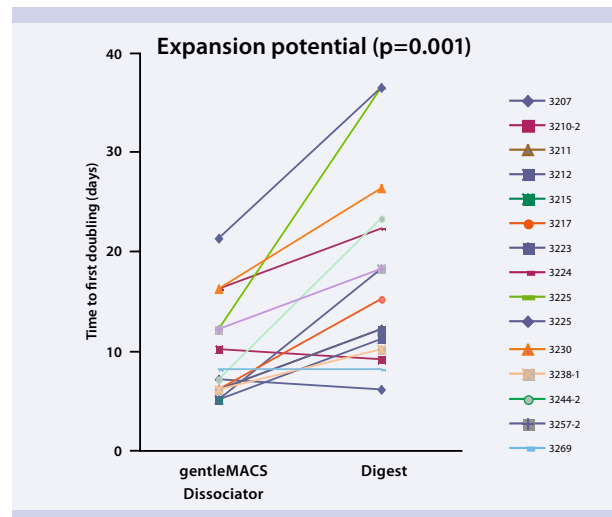


Figure 2 Expansion potential of TIL generated by using the gentleMACS Dissociator or overnight digestion. For comparison, samples from 15 patients were processed using the gentleMACS Dissociator or overnight digestion. Expansion of TIL was measured as described in Materials and methods.

Results

The initial gentleMACS Dissociation product, when compared to that of the current clinical method of overnight enzymatic digestion, showed no significant difference in the cellular composition, the yield of viable cells, or the overall viability. (fig. 1)

For both methods, the initial dissociation products could be efficiently expanded in culture and were functional *in vitro* and *in vivo*. There was similar eventual TIL generation and TIL attributes in most respects. Results do show, however, that the resulting TIL cultures from the standardized, semi-automated protocol using the gentleMACS Dissociator expanded significantly faster than those from manually dissociated tumors (fig. 2). Further analysis of these expanded TIL showed full functionality of the cells *in vitro*. There were few evaluable samples, but there was no obvious difference in function between cells obtained through the gentleMACS Procedure or standard digest (table 1).

There was no significant difference in lymphocyte subsets of the resulting TIL. 12 of 16 TIL cultures derived from the gentleMACS Protocol had more CD8⁺ cells than those of a standard digest, but this trend was not statistically significant. There was also a slight trend towards more CD4⁺ cells in the TIL of a standard digest (data not shown).

The optimized gentleMACS Dissociation programs were compared with the standard digest method for clinical expansion and efficacy. The cultures derived from the gentleMACS Protocol expanded well (fig. 3) and caused tumor regression in patients when used in melanoma immunotherapy protocols (fig. 4).

	Melanoma cell line						FrTu						
	None	A2-		A2+		3104	3210	3003	3210-2	3217	3089	3010-2	3257-1
		888	938	526	624								
		A1, 24	A1, 24	A2, 3	A2, 3	A11, 26	A2, 3	A1, 2	A2, 30	A24, 68	A1, 0201	A 30, 33	A 24, 32
TIL 3207 (A1, A30) Std. Digest d22	322	247	390	45									
TIL 3207 (A1, A30) gentleMACS d23	78	443	861	116									
TIL 3211 (A1, A3) Std. Digest d24	29	23	18	20	33	46	61						
TIL 3211 (A1, A3) gentleMACS d25	40	253	119	69	150	59	67						
TIL 3212 (A1, A2) Std. Digest d19	46	82	658	488	322	88		353					
TIL 3212 (A1, A2) gentleMACS d20	23	191	482	328	284	97		322					
TIL 3210-2 (A1, A30) Std. Digest d28	45	34	33	20	33	44			141				
TIL 3207 (A1, A30) gentleMACS d28	6	28	23	11	21	97			315				
TIL 3223 (A2) Std. Digest d15	19	93	154	>32890	>17980								
TIL 3223 (A2) gentleMACS d16	14	71	112	>38210	>27440								
TIL 3217 (A24, A68) Std. Digest d21	15	332	180	87	289					424	151	93	
TIL 3217 (A24, A68) gentleMACS d22	11	206	134	37	131					298	69	92	
													17
													133

Table 1 Function of TIL generated by using the gentleMACS Dissociator or overnight digestion. Function of TIL was evaluated by coculturing TIL with melanoma cell lines or primary melanoma cells (FrTu) and analysis of IFN-γ release by TIL. Numbers indicate IFN-γ concentrations in pg/mL. Bold numbers indicate results that were significantly different from the controls that included no tumor cells.

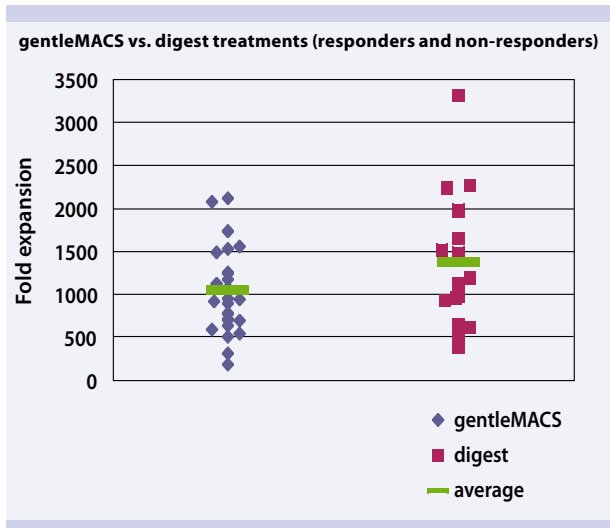


Figure 3 Clinical-scale expansion of TIL generated by using the gentleMACS Dissociator or overnight digestion. TIL generated by the gentleMACS Procedure or the standard digest were expanded to clinically relevant cell numbers. The analysis included both TIL that resulted in an objective clinical response and TIL that did not induce a response.

Fisher's exact test:		
	OR	NR
gentleMACS Dissociator	10 (38%)	16
digest	7 (41%)	10

Figure 4 Efficacy of TIL generated by using the gentleMACS Dissociator or overnight digestion. Fisher's exact test was performed on the percentage of objective responses (OR) to compare efficacies of TIL generated by the gentleMACS Procedure or overnight digestion (p= 0.69).

Conclusion

An optimal SOP for the preparation of TIL using the gentleMACS Dissociator was established. The gentleMACS Dissociator from Miltenyi Biotec provides a semi-automated protocol for the dissociation of melanoma tissue leading to single-cell suspensions with yields and viabilities comparable to that of overnight enzymatic digestion. Both methods provide similar initial TIL/tumor cell ratios, viability, and total cell yield for most tumor tissue samples. However, TIL from gentleMACS Dissociations showed significantly enhanced initial expansion in culture. The gentleMACS Procedure is faster, more standardized, and more efficient for tumor samples up to 5 grams, whereas we have found that the standard digest is more convenient for processing larger tumors. Samples prepared using the gentleMACS Dissociator were efficiently expanded to clinically relevant cell numbers ($\sim 5.0 \times 10^{10}$) and were able to mediate tumor regression in advanced melanoma patients.

Based on our results, we have incorporated the gentleMACS Dissociator into our clinical SOPs and use it interchangeably with the current overnight enzymatic digestion for the generation of TIL for adoptive cell therapy trials.

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References

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