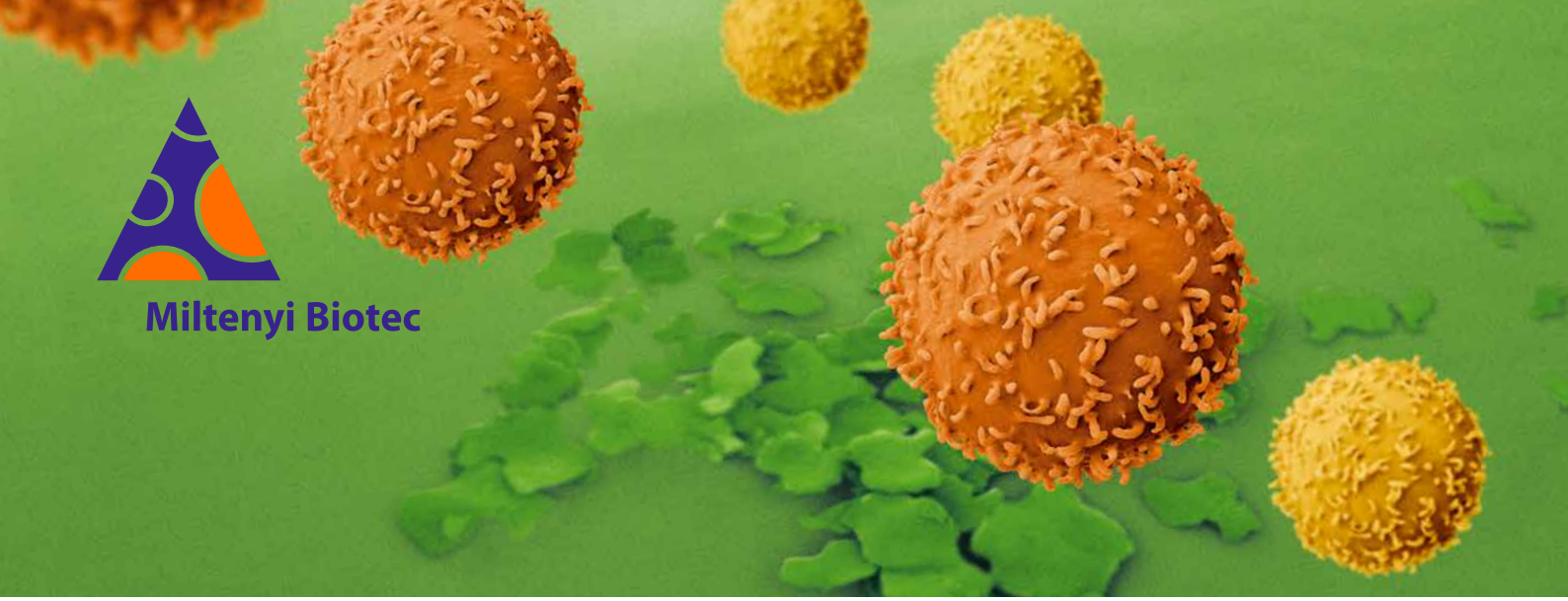

Miltenyi Biotec

# Analysis of FoxP3$^{+}$ regulatory T cells from mouse dorsal skin

**Natalie Groß, Katja Bieber, and Ralf Ludwig**
Lübeck Institute of Experimental Dermatology,
University of Lübeck, Lübeck, Germany

## Background

Regulatory T cells (Tregs) play an important role in the control of autoimmune skin diseases. Epidermolysis bullosa acquisita (EBA) is a blistering autoimmune dermatosis that belongs to the group of pemphigoid diseases (PDs) and it is characterized by the development of autoantibodies against type VII collagen[1]. During the effector phase of PDs a network of different lymphocyte populations regulate the recruitment and activation of myeloid cells. Among these different lymphocyte subsets, Tregs are known to play disease-suppressing local roles[2,3]. Previous results indicate new promising therapeutic alternatives for PDs in respect to Tregs. However, the mechanisms of how Tregs serve a protective function in PDs are still largely unknown[4–6].

Experimental EBA mouse models enable examination of the function of Tregs in the pathogenesis of the disease. A deeper investigation requires the dissociation of the mouse skin to generate single-cell suspensions for downstream flow cytometric analysis. Mouse skin dissociation can be challenging when working with manual procedures that can lead to high user-dependent variability and low numbers of viable target cells.

In this application note, we describe a standardized method for the dissociation of mouse dorsal skin and subsequent flow cytometric analysis of Tregs. This method is based on the use of the Multi Tissue Dissociation Kit 1 in combination with the gentleMACS™ Octo Dissociator with Heaters.

## Materials and methods

**Extraction of mouse dorsal skin**

Whole skin samples were obtained from euthanized healthy C57BL/6 wild type mice. The dorsal skin was shaved (excluding the flanks) and a depilatory cream was applied to remove any remaining fur. The depilated skin was cleaned with water and alcohol before extraction. The mice were used as approved by the governmental ethics committee for animal welfare.

**Tissue dissociation**

Dissociation of the skin was performed using either a manual protocol or an automated protocol based on gentleMACS Technology (gentleMACS Protocol):

- Manual protocol: In a petri dish, 0.24 g of skin were cut with scalpels into pieces of about 2 mm and submerged in 4 mL of RPMI 1640 medium supplemented with L-Glutamine (Lonza Lot:7MB037) and containing Liberase™ TM Research Grade (Roche) at a final concentration of 0.4 mg/mL (Liberase medium). The tissue pieces and the Liberase medium were transferred into a 15 mL tube and incubated in an incubator with 5% $CO_2$ at 37 °C for 30 minutes and were vortexed every 5 minutes. After incubation, an equal amount of RPMI 1640 medium was added to inactivate the Liberase medium. The cell suspension was then filtered using a 70 µm cell strainer, and 3 mL of RPMI 1640 medium was used to wash the cell strainer. The filtrated cell suspension was centrifuged for 5 minutes at 380×g at 4 °C. The supernatant was discarded and the cell pellet was resuspended in PBS for flow cytometric analysis.

- gentleMACS™ Protocol: In a petri dish, 0.6 to 1 g of skin was cut with scalpels into pieces of about 2 mm and submerged in 4.6 mL RPMI 1640 medium. The tissue pieces and the RPMI medium were transferred into a gentleMACS C Tube. The enzymes of the Multi Tissue Dissociation Kit 1 were reconstituted according to the instructions from the data sheet and added to the C Tube in the following amounts: 300 µL Enzyme D, 50 µL Enzyme R, and 25 µL Enzyme A. Dissociation was performed using a customized program* with the gentleMACS Octo Dissociator with Heaters. The duration of the customized program is 3 hours. After dissociation, a short spin at 300×g was performed to collect all cells. The cell pellet was resuspended and filtered using a 70 µm MACS® SmartStrainer, 5 mL of RPMI medium was used to wash the SmartStrainer. The filtrated cell suspension was centrifuged at 300×g for 10 minutes, the supernatant was discarded, and the cell pellet was resuspended in PBS for flow cytometric analysis.

VISIT

*This customized program is available upon request. For more information please contact

▸ **miltenyibiotec.com/support**

### Flow cytometric analysis

Cells were labeled with the Viobility™ 405/520 Fixable Dye to differentiate dead from viable cells. Next, analysis of Tregs was performed by staining the surface marker CD4 using the CD4 Antibody, anti-mouse, VioBlue®, REAfinity™, and subsequently intracellular staining of FoxP3 was performed using the FoxP3 Staining Buffer Set and FoxP3 Antibody, anti-mouse, APC, REAfinity. Samples were acquired using a MACSQuant® Analyzer 10.

## Results

We compared two methods to dissociate dorsal skin from C57BL/6 mice, a manual protocol based only on enzymatic digestion and an automated method based on gentleMACS Technology, which combines both enzymatic and mechanical dissociation. The average number of total viable cells obtained after dissociation were comparable between both protocols, but the gentleMACS protocol showed lower variability between samples and more reproducibility (fig. 1A). Furthermore, higher numbers of Tregs ($CD4^+FoxP3^+$) were obtained when using the gentleMACS protocol (fig. 1B and 2).

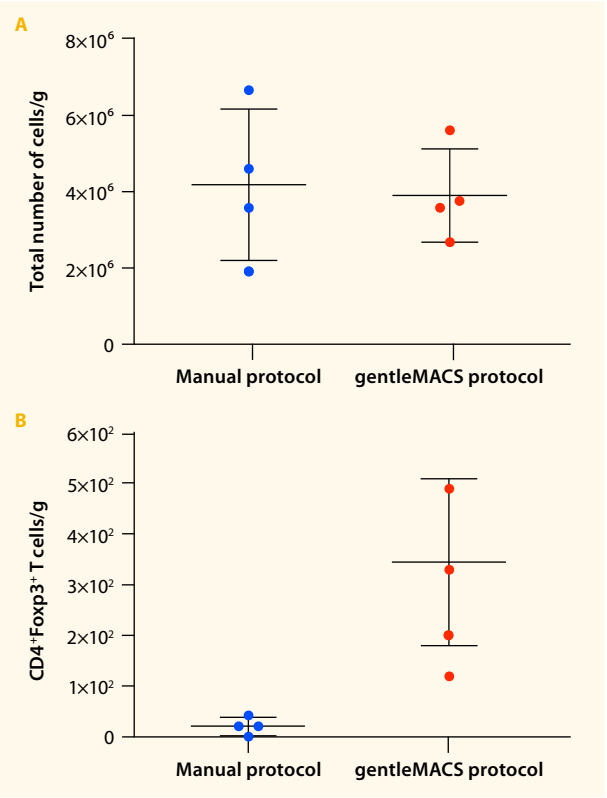


**Figure 1: Analysis of Tregs after dissociation of mouse dorsal skin using a manual protocol or the gentleMACS protocol.**
(A) Total numbers of viable cells after dissociation, per gram of skin. (B) Number of viable $CD4^+FoxP3^+$ Tregs per gram of skin.

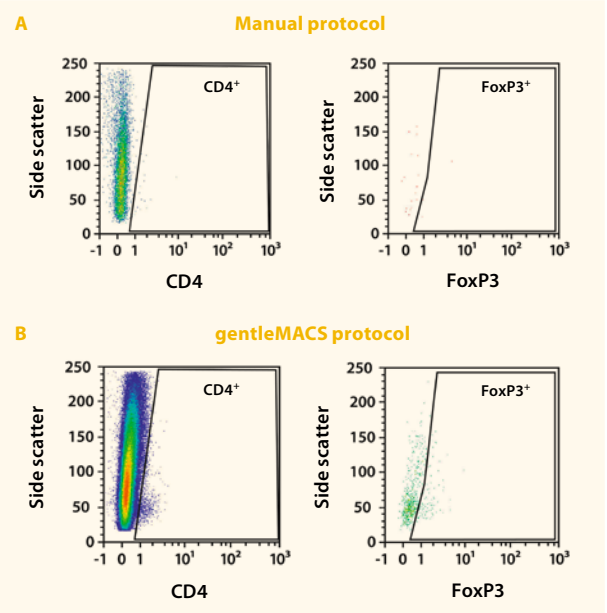


**Figure 2: Flow cytometric analysis of $CD4^+FoxP3^+$ Tregs from mouse dorsal skin.** Representative dot plots showing $CD4^+FoxP3^+$ Tregs, pre-gated on viable cells. (A) Cells obtained using a manual dissociation protocol, and (B) using the gentleMACS Protocol.

## Conclusions

- The Multi Tissue Dissociation Kit 1 in combination with the gentleMACS™ Octo Dissociator with Heaters (gentleMACS Protocol), enables fully automated and efficient dissociation of mouse dorsal skin to obtain viable single-cell suspensions.
- The gentleMACS Protocol represents a standardized method to reproducibly and easily obtain consistently higher numbers of Treg cells from mouse dorsal skin. Furthermore, this protocol ensures epitope preservation for reliable downstream flow cytometric analysis of Treg cells.

| MACS Product | Order no. |
|---|---|
| gentleMACS Octo Dissociator with Heaters | 130-096-427 |
| Multi Tissue Dissociation Kit 1 | 130-110-201 |
| MACS Smart Strainer (70 µm) | 130-098-462 |
| FoxP3 Staining Buffer Set | 130-093-142 |
| Viobility 405/520 Fixable Dye | 130-109-814 |
| CD4 Antibody, anti-mouse, VioBlue, REAfinity | 130-118-568 |
| FoxP3 Antibody, anti-mouse, APC, REAfinity | 130-111-601 |
| MACSQuant Analyzer 10 | 130-096-343 |

VISIT

For antibodies conjugated to a wide range of fluorochromes visit

▸ **miltenyibiotec.com/antibodies**

## References


1. Witte, M. *et al.* (2016) Discovering potential drug-targets for personalized treatment of autoimmune disorders – what we learn from epidermolysis bullosa acquisita. Exp opinion on therap targets. 20(8):985–98.
2. El Beidaq, A. *et al.* (2016) *In Vivo* Expansion of Endogenous Regulatory T Cell Populations Induces Long-Term Suppression of Contact Hypersensitivity. J Immunol. 197:1567–76.
3. Hirose, M. *et al.* (2016) Reduced skin blistering in experimental epidermolysis bullosa acquisita after anti-TNF treatment. Mol Med. 22:918–26.
4. Bieber, K. *et al.* (2017) Analysis of serum markers of cellular immune activation in patients with bullous pemphigoid. Exp Dermatol. 26:1248–52.
5. Witte, M. *et al.* (2016) Discovering potential drug-targets for personalized treatment of autoimmune disorders - what we learn from epidermolysis bullosa acquisita. Exp Opinion on Therap Targets. 20:985–98.
6. Kulkarni, U. *et al.* (2016) IL-10 mediates plasmacytosis-associated immunodeficiency by inhibiting complement-mediated PMN migration. J Allergy Clin Immunol. 137:1487–97 e6.



Miltenyi Biotec

**Miltenyi Biotec B.V. & Co. KG** | Phone +49 2204 8306-0 | Fax +49 2204 85197 | macsde@miltenyi.com | **www.miltenyibiotec.com**

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.





V.01