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Regulation of skin tissue-resident memory T cells in hematopoietic stem cell transplantation and acute graft-versus-host disease

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

Submitted by

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Declaration

This doctoral thesis is organized in a cumulative format, with one manuscript published in the *Journal of Experimental Medicine* (Strobl, Gail et al, 2021) and one currently submitted manuscript (Gail, Deckert et al, 2024). I, Laura Marie Gail, am shared-first author of both original manuscripts and the sole author of this thesis. The thesis also includes a first-author review article in *Frontiers in Immunology* (Gail et al, 2023), that is referenced in the introduction and attached in the Appendix of this thesis.

Included publications:

J Strobl*, **LM Gail***, L Kleissl, R Vinay Pandey, V Smejkal, J Huber, V Puxkandl, L Unterluggauer, R Dingelmaier-Hovorka, D Atzmüller, T Krausgruber, C Bock, P Wohlfarth, W Rabitsch, G Stary.

Human resident memory T cells exit the skin and mediate systemic Th2-driven inflammation

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Human type 17 tissue-resident memory T cells are dysfunctional in type 1-driven skin inflammation

submitted manuscript (*shared first authorship)

LM Gail, KJ Schell, P Lacina, J Strobl, SJ Bolton, E Steinbakk Ulriksen, K Bogunia-Kubik, H Greinix, RE Crossland, M Inngjerdigen, G Stary.

Complex interactions of cellular players in chronic Graft-versus-Host Disease

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Most of the work described in this thesis was carried out at the Department of Dermatology, Medical University of Vienna, and the CeMM Center for Molecular Medicine of the Austrian Academy of Sciences, in the lab of the thesis supervisor, Univ.-Prof. Dr. Georg Stary. Patient and healthy control samples were obtained from the Department of Dermatology, Department of Plastic and Reconstructive Surgery and Department of Medicine I at the General Hospital Vienna, Medical University of Vienna. All research performed on human samples in the studies included in this thesis is in line with national law and the corresponding ethics approval licenses granted by the ethics committee of the Medical University of Vienna are listed in the individual manuscripts.

The manuscripts also contain detailed descriptions of the individual contributions of all co-authors in the “Author contributions” section, as well as their affiliations. The support from core facilities of the Medical University of Vienna and at the CeMM is also acknowledged in the individual manuscripts. Furthermore, the Acknowledgment section of every manuscript lists the relevant funding sources that supported the respective work.

I declare that this thesis does not contain AI-generated text passages, except the translation of the English abstract to German language (pages viii-ix). I translated the abstract using Perplexity AI and subsequently proof-read and adapted this translation to avoid misinterpretations. Word for Microsoft 365 tools “Spelling and Grammar” and “Thesaurus” were used for text proofing for the entire thesis. Endnote X9 was used to format and manage citations.

Permission to reprint the published manuscripts (Strobl, Gail et al, 2021, Gail et al, 2023) in this thesis is granted under a Creative Commons License by the publishers Rockefeller University Press and Frontiers, respectively. Figures included in the introduction of this thesis were generated by me, using Biorender software under a paid subscription in my name and publication licenses for each figure are attached in the Appendix of this thesis.

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Abstract (English)

Tissue-resident memory T cells (TRM) are a specialized subset of memory T cells that reside permanently in non-lymphoid organs, such as the skin. They are instrumental for orchestrating tissue homeostasis and providing localized immunity at barrier sites. However, they are also implicated in driving inflammatory skin diseases. Despite their significance, studies in humans addressing heterogeneity, developmental and conserved pathways, and disease-specific roles of TRM remain limited.

This thesis focuses on TRM biology in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT). Our studies build on previous work from our lab, which demonstrated that host TRM survive myeloablative conditioning and persist in the skin for years after transplantation alongside transplanted donor-derived T cells. Importantly, higher numbers of pre-existing host TRM before transplantation are associated with the development of acute graft-versus-host disease (aGVHD), a T cell-mediated inflammatory disorder, affecting many HSCT patients and commonly manifesting in the skin. We hypothesized that dissecting the biology of host and donor TRM after transplantation, especially during aGVHD, could reveal important principles of TRM behavior.

Recently, blood re-entry of bona fide skin TRM from skin was proposed as a concept to distribute tissue immunity across larger distances. To investigate whether human skin TRM can give rise to circulating T cell progeny and study the functional implications of this, we performed sampling of skin and blood from HSCT patients at different timepoints before and after transplantation, and at the onset of aGVHD. Using cutting-edge technologies such as RNA sequencing, T cell receptor (TCR) sequencing, mathematical modeling, *in situ* genotyping, and multiparametric flow cytometry, we could demonstrate the presence of skin TRM-derived circulating T cells (cTRM) in blood after HSCT and in aGVHD. These cTRM exhibited pro-inflammatory properties and expressed receptors facilitating migration to other aGVHD target organs, suggesting a potential role in systemic inflammation.

In our second study, we employed a similar sampling scheme and performed single-cell RNA and TCR sequencing of serial, matched skin and blood samples. Subsequent computational analysis allowed us to separately trace individual host and donor T cells at tissue-, time-, clonotype- and phenotype resolution. We uncovered that in aGVHD a striking shift to type 1 polarized inflammatory profiles coincided with the specific reduction and functional impairment of type 17 polarized TRM, suggesting that distinct molecular networks govern the development and maintenance of type 1- versus type 17-polarized TRM.

Overall, these findings provide important insights into the regulation and function of human skin TRM, with potential implications for understanding and treating inflammatory skin diseases driven by TRM dysregulation.

Abstract (German)

Gewebeständige Gedächtnis-T-Zellen (TRM) sind eine Untergruppe von Gedächtnis-T-Zellen, die dauerhaft in nicht-lymphatischen Organen wie der Haut verbleiben. Sie spielen eine entscheidende Rolle bei der Erhaltung der Gewebekomöostase und der Immunabwehr in Barrieregeweben. Gleichzeitig sind sie jedoch auch an der Pathogenese entzündlicher Hautkrankheiten beteiligt. Bislang fehlen adäquate und umfassende Untersuchungen am Menschen, welche die heterogenen Phänotypen und Funktionen von TRM, ihre Entwicklungs- und Erhaltungsabhängigkeiten, sowie ihre Rolle bei gewebespezifischen Krankheitsprozessen untersuchen.

In dieser Dissertation untersuchten wir die Biologie von TRM bei Patienten, die sich einer allogenen hämatopoetischen Stammzelltransplantation (HSCT) unterzogen. Frühere Arbeiten unseres Labors hatten gezeigt, dass TRM die myeloablative Konditionierung überstehen und jahrelang neben Spender-T-Zellen in der Haut verbleiben können. Interessanterweise ist das Vorhandensein von TRM vor der Transplantation mit der Entwicklung einer akuten Graft-versus-Host-Krankheit (aGVHD) assoziiert. Die aGVHD ist eine T-Zell-vermittelte entzündliche Erkrankung, die viele HSCT-Patienten betrifft und sich häufig an der Haut manifestiert. Wir stellten die Hypothese auf, dass eine getrennte Analyse von Empfänger- und Spender-TRM nach der HSCT und bei aGVHD zentrale Prinzipien der TRM-Heterogenität, -Entwicklung, -Erhaltung und deren Migrationsmuster aufdecken würde.

Neueste Studien postulieren, dass Haut-TRM wieder in den Blutkreislauf eintreten können, um die Gewebeimmunität über größere Entfernungen zu koordinieren. Um zu untersuchen, ob TRM der menschlichen Haut zirkulierende T-Zellen hervorbringen können, und um die funktionellen Auswirkungen davon zu studieren, entnahmen wir Haut- und Blutproben von HSCT-Patienten vor und nach der Transplantation sowie zu Beginn der aGVHD. Mithilfe moderner Technologien wie RNA-Sequenzierung, T-Zell-Rezeptor (TCR)-Sequenzierung, mathematischer Modellierung, *In-situ*-Genotypisierung und multiparametrischer Durchflusszytometrie konnten wir die Existenz zirkulierender T Zellen mit gewebsähnlichen Eigenschaften (cTRM) im Blut, sowie deren Abstammung von Haut-TRM nach HSCT und bei aGVHD nachweisen. Die pro-entzündlichen Eigenschaften und die Expression von Rezeptoren zur Rekrutierung in aGVHD-Zielorgane unterstreichen die potenzielle Rolle der cTRM bei der systemischen Ausbreitung von Entzündungen.

In unserer zweiten Studie führten wir Einzelzell-RNA- und TCR-Sequenzierungen an seriellen Haut- und Blutproben derselben Patientengruppe durch. Unsere Analysen ermöglichte uns, einzelne Wirts- und Spender-T-Zellen auf Gewebe-, Zeit-, Klonotyp- und Phänotyp-Ebene getrennt zu verfolgen. Wir entdeckten, dass bei aGVHD eine auffällige Verschiebung zu Typ-1-polarisierten Entzündungsprofilen mit einer spezifischen Reduktion und funktionellen Beeinträchtigung von Typ-17-polarisierten TRM einherging. Dies deutet darauf hin, dass

unterschiedliche molekulare Netzwerke die Entwicklung und Erhaltung von Typ-1- gegenüber Typ-17-polarisierten TRM steuern.

Diese Ergebnisse liefern wichtige Einblicke in die Regulierung und Funktion menschlicher Haut-TRM und bieten neue Ansätze für die Erforschung und Behandlung entzündlicher Hauterkrankungen, bei denen TRM eine zentrale Rolle spielen.

Publications arising from this thesis

Included in this thesis, as described in the declaration, are two published articles and one submitted manuscript:

J Strobl*, **LM Gail***, L Kleissl, R Vinay Pandey, V Smejkal, J Huber, V Puxkandl, L Unterluggauer, R Dingelmaier-Hovorka, D Atzmüller, T Krausgruber, C Bock, P Wohlfarth, W Rabitsch, G Stry

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Human type 17 tissue-resident memory T cells are dysfunctional in type 1-driven skin inflammation

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Complex interactions of cellular players in chronic Graft-versus-Host Disease

Front Immunol. June 2023, 14:11999422. doi: 10.3389/fimmu.2023.1199422. (Review)

Additionally, during the doctoral studies I contributed to several collaborative projects, resulting in co-authorship on the following published manuscripts, which are not officially part of this thesis, but contributed to the topic and are cited accordingly throughout the introduction and discussion:

S Saluzzo, R Vinay Pandey, **LM Gail**, R Dingelmaier-Hovorka, L Kleissl, L Shaw, B Reininger, D Atzmüller, J Strobl, V Touzeau-Römer, A Beer, C Staud, A Rieger, M Farlik, W Weninger, G Stingl, G Stry

Delayed antiretroviral therapy in HIV-infected individuals leads to irreversible depletion of skin- and mucosa-resident memory T cells

Immunity. December 2021, 54(12):2842-2858.e5. doi: 10.1016/j.immuni.2021.10.021

S Saluzzo, **LM Gail**, T Neuwirth, R Vinay Pandey, M Farlik, G Stry

Processing human skin samples for single-cell assays

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N Bayer, R Vinay Pandey, B Hausman, F Deckert, **LM Gail**, J Strobl, P Pjevac, C Krall, L Unterluggauer, A Redl, V Bachmayr, L Kleissl, M Nehr, R Kirkegaard, A Makristathis, M

Watzenböck, R Nica, C Staud, L Hammerl, P Wohlfarth, R Eckert, S Knapp, W Rabitsch, D Berry, G Stry

Disturbances in microbial skin recolonization and cutaneous immune response following allogeneic stem cell transfer

Leukemia. November 2022, 36(11):2705-2714. doi: 10.1038/s41375-022-01712-z.

RV Pandey, J Strobl, A Redl, L Unterluggauer, **LM Gail**, L Kleissl, S Müller, D Atzmüller, V Fife-Gernedl, T Krausgruber, H Knaus, M Mitterbauer, P Wohlfarth, W Rabitsch, C Bock, G Stry.

Epigenetic regulation of T cell lineages in skin and blood following hematopoietic stem cell transplantation

Clin Immunol, March 2023, 248(5):109245. doi: 10.1016/j.clim.2023.109245.

J Strobl, **LM Gail**, L Krecu, S Madad, L Kleissl, L Unterluggauer, A Redl, K Brazdilova, S Saluzzo, P Wohlfarth, HA Knaus, M Mitterbauer, W Rabitsch, M Haniffa, G Stry.

Diverse macrophage populations contribute to distinct manifestations of human cutaneous graft-versus-host disease

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P Starkl, G Jonsson, T Artner, B Lenfers Turnes, **LM Gail**, T Oliveira, A Jain, N Serhan, K Stejskal, K Lakovits, A Hladik, M An, KM Channon, H Kim, T Köcher, W Weninger, G Stry, S Knapp, V Klang, N Gaudenzio, CJ Woolf, S Tikoo, R Jain, JM Penninger, SJF Cronin

Mast cell-derived BH4 and serotonin are critical mediators of postoperative pain

Sci Immunol, August 2024, 98(9):eadh0545. doi: 10.1126/sciimmunol.adh0545

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I Saridakis, M Schupp, H Zhang, T Leischner, **LM Gail**, M Drescher, F Doubek, K Günther, D Kaiser, G Stry, N Maulide

Synthesis and Immunological Evaluation of (-)-FR252921 and Novel Analogs against Primary Human Immune Cells

Submitted manuscript

J Strobl, L Kleissl, S Conolly, **LM Gail**, S Weninger, T Frey, A Kopf, J Eder, M Markowicz, P Barikova, C Freystätter, K Schmetterer, H Strobl, H Stockinger, M Wijnveld, G Stry

Langerhans cells induce tolerance and drive immune evasion during tick-borne pathogen transmission in human skin

Submitted manuscript

Abbreviations

AD	Atopic Dermatitis
AlloBMT	Allogeneic bone marrow transfer
APC	Antigen presenting cell
CAR	Chimeric antigen receptor
(c)DC	(Conventional) dendritic cell
cTRM	Circulating T cells with tissue-resident memory phenotype
DETC	Dendritic epidermal T cell
FISH	Fluorescence in situ hybridization
FRC	Fibroblastic reticular cells
(a/c) GVHD	(Acute/chronic) graft-versus-host disease
GI GVHD	Gastrointestinal GVHD
GVL	Graft-versus-leukemia effect
HCA	Human cell atlas
HLA	Human leukocyte antigen
HIV	Human immunodeficiency virus
HSCT	Allogeneic hematopoietic stem cell transplantation
HSV	Herpes simplex virus
ILC	Innate lymphoid cell
LC	Langerhans cell
LCMV	Lymphocytic choriomeningitis virus
LTi	Lymphoid tissue inducer cell
MHC	Major histocompatibility complex
NK cell	Natural killer cell
PRR	Pattern recognition receptor
SI	Small intestine

SLO	Secondary lymphoid organ
SNP	Singel nucleotide polymorphism
TCM	Central memory T cell
TCR	T cell receptor
Tc(1/17) cell	CD8+ T cell with type 1/type 17 polarization
TEM	Effector memory T cell
Th(1/2/17)	CD4+ T helper cell with type 1/type 2/type 17 polarization
Treg	CD4+ regulatory T cell
TRM(1/17)	Tissue-resident memory T cell with type 1/type 17 polarization

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I am immensely grateful to my shared-first co-authors on my thesis papers, Johanna and Florian. I enjoyed discussing and sharing ideas with you and I strongly believe that combining our efforts has not only produced higher-quality output, but also made the process much more fun. Also big thanks to all co-authors that contributed to the projects.

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1. Introduction

This thesis is concerned with the cutaneous immune system. The skin as a barrier organ has key functions in host defense and a plethora of different immune cells reside in the skin to orchestrate immunity and tissue homeostasis. The projects that are part of this thesis aimed to better understand phenotypic and functional heterogeneity of one specific immune cell population of the skin, so-called tissue-resident memory T cells (TRM). We studied these cells in human skin, in a special cohort of patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT). These samples allowed us to investigate TRM homeostatic generation and maintenance, as well as their role in inflammatory settings, when patients develop cutaneous acute graft-versus-host disease (aGVHD).

In chapter 1.1. of the introduction, I will briefly describe skin anatomy and introduce the different immune cell subsets and their broad functions. TRM will be introduced in detail in chapter 1.2., describing current knowledge on their development and maintenance, regarding transcriptional regulation, metabolic requirements and migratory behavior. I will give an overview of their protective functions in infection and tumor control, as well as their pathogenic potential in several inflammatory skin diseases. There will also be a brief section on commonly used pre-clinical models, which will lead me to explain why we chose to study human TRM in HSCT and aGVHD patients. In chapter 1.3., I will describe the clinical characteristics of HSCT and aGVHD patients, explain the pathophysiology of aGVHD with a special focus on the role of T cells, particularly the TRM population. Lastly, in chapter 1.4., I will outline the specific aims of this thesis.

1.1. The anatomy and function of human skin as an immunological barrier organ

Human skin is a crucial barrier organ. It has wide-ranging functions that enable our interaction with, and protection from the environment. This includes sensation, thermoregulation, regulation of water loss, protection from UV radiation, synthesis of Vitamin D, and importantly, immune defense and preventing entry of harmful agents. These different functions are mediated through several physical, structural, and chemical properties of the skin, and have been reviewed extensively¹⁻⁴. In the following chapters, I will briefly summarize the anatomy of human skin and the different components that mediate its broad physiological functions, and then focus on the skin as an immunological organ, introducing cellular players of the skin immune system. Figure 1 illustrates an overview of the main functions and immune cells in healthy human skin, which are important to mediate organ homeostasis and host defense.

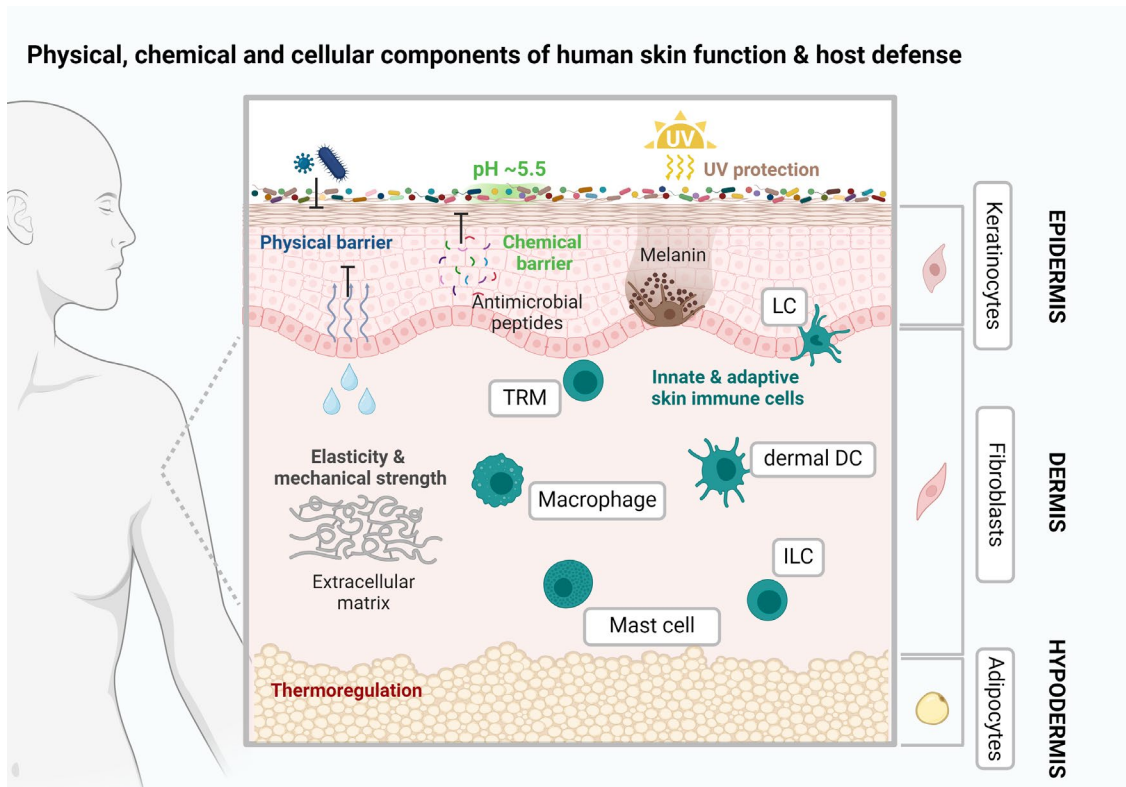


Figure 1 – Physical, chemical and cellular components of human skin function and host defense

Simplified schematic of skin anatomy, the major properties mediating organ homeostasis and host defense and the different immune cell subsets present in healthy human skin. TRM = Tissue-resident memory T cell, LC = Langerhans cell, dermal DC = dermal dendritic cell, ILC = innate lymphoid cell. Note that skin appendages, such as hair follicles, sweat glands or sensory structures, as well as vasculature are not depicted in the schematic, and immune cells of the hypodermis are not shown.

1.1.1. Anatomy and function of human skin

Different skin layers and their components

The anatomical organization of human skin is complex and comprises highly specialized cells that can sense and process cues from the environment and communicate with each other to orchestrate skin homeostasis^{1,3}. Broadly, the skin can be divided into three different layers. The hypodermis is the lowest skin layer with subcutaneous fat and connective tissue, as well as blood vessels, lymph vessels and nerves. It serves as an energy depot and metabolic regulation hub and has functions in thermoregulation¹. In the middle layer, the dermis, fibroblasts produce extracellular matrix proteins to form connective tissue that confers elasticity and strength. The dermis also harbors different skin appendages, such as sweat and sebaceous glands, hair follicles, different nerve structures, and a large network of blood and lymph vessels¹. The epidermis is the outermost layer. It is non-vascularized and built up by keratinocytes in different stages of development that define sublayers within the epidermis.

These sublayers include the basal layer, with stem-cell like, renewing keratinocytes, the stratum spinosum with dividing keratinocytes, and the stratum granulosum, where keratinocytes produce keratin and start to synthesize lipids and proteins to form the cornified envelope of dead keratinocytes (corneocytes) in the stratum corneum³. Melanocytes, the pigment-producing cells of the human body, reside in the basal layer of the epidermis and the hair follicles and are responsible for coloring of skin and hair and mediating UV protection through production of melanin².

Physical and chemical host defense mechanisms in the skin

The outermost layer of the skin, the epidermal stratum corneum, serves as a physical protection shield. It is formed of dead corneocytes that are glued together by different types of lipids, creating a barrier for water loss and entry of microbes or noxious agents³. In the underlying layers of the epidermis, containing live keratinocytes, cells are connected by tight junctions, allowing only limited passage between them, and providing mechanical strength to protect from physical trauma. Keratinocytes have additional properties to facilitate skin protection, such as the production of antimicrobial peptides, which can prevent colonization with certain harmful microbes⁴. Another mechanism of host defense is the acidic pH of the skin, generated by sweat and sebum, as well as through the activity of skin resident microbiota. The skin pH favors the colonization with beneficial microbes, while preventing the growth of certain harmful ones⁵. Besides physical and chemical properties, the skin is equipped with a dense network of immune cells, which will be described in the next chapter.

1.1.2. The skin immune system

Different types of specialized immune cells can be found in all layers of the skin, and they fulfill different functions depending on their localization and their inherent properties (reviewed by Kabashima et al¹). While skin pH, mechanical properties, and host defense mechanisms of non-immune cells in the skin are important for protection and maintenance of homeostasis, the skin immune system is indispensable. Without functioning localized skin immunity, infections cannot be cleared, and tissue repair cannot be properly orchestrated in case of injury. Therefore, the immune cells are an integral part of skin organ function. In this chapter, the major immune cell types and their functions in the skin are summarized.

Innate immune cells - first-line responders in the skin

The skin contains resident cells of the innate immune system that can rapidly respond in case of an insult¹. Skin resident macrophages reside in the dermis and in the subcutaneous adipose tissue. Their main functions include phagocytosis of dead cells and microbes and regulation of immune responses by sensing of harmful agents, antigen presentation to adaptive immune cells, and production of an array of cytokines and chemokines. Macrophages are also crucial for tissue remodeling and wound healing through the production of cytokines, growth factors

and enzymes, as well as through phagocytosis of cellular debris (reviewed by Mass et al⁶). Macrophages can adapt diverse polarizations, and while they were classically divided into pro-inflammatory M1 and immune-regulatory M2 subsets, this is oversimplified and outdated as macrophage phenotypes are much more granular and fine-tuned *in vivo*⁶.

Mast cells are innate cells that reside in the dermis and hypodermis. As granulocytes, mast cells contain granules with pre-formed mediators they can rapidly secrete in case of activation during inflammation. Skin mast cells release vast amounts of histamine, majorly contributing to allergic reactions in the skin. They can also release various inflammatory mediators, tissue remodeling enzymes and react to neuropeptides and co-factors that regulate sensory reactions in the skin⁷⁻⁹.

Another group of innate cells in the skin are innate lymphoid cells (ILC). These cells have only been recently discovered and have functions mirroring adaptive lymphocytes. However, they are not antigen specific. ILC comprise group 1 Natural Killer (NK) cells and ILC1, ILC2 and group 3 ILC3 and lymphoid tissue inducer cells (LTi) (reviewed by Vivier et al¹⁰). ILC1,2 and 3 have cytokine profiles corresponding to those of T helper 1, T helper 2 and T helper 17 cells respectively, whereas LTi produce high amounts of lymphotoxin and are important for formation of secondary lymphoid structures¹⁰. In healthy human skin ILC2 and ILC3 predominate^{11,12}, and have a distinct proteome from their blood counterparts and skin T cells¹¹. Functionally, they are implicated in host defense, wound healing and tissue homeostasis¹³. During inflammatory responses a variety of innate cells are recruited to the tissue, such as neutrophils, eosinophils, monocytes, or NK cells, depending on the inflammatory or infectious trigger.

Antigen presentation – bridging innate and adaptive immunity

While antigen presenting cells (APC) are regarded as part of the innate immune system and are equipped with similar functions as other innate cells, such as pathogen sensing and production of cytokines, they are also the key mediators between innate and adaptive immune responses (reviewed by Neuwirth et al¹⁴). APC take up antigens, migrate to secondary lymphoid organs (SLO) and initiate antigen-specific activation and expansion of adaptive immune cells (T cells and B cells), leading to formation of immune memory.

Although macrophages and other cell populations can present antigens, the most proficient cells for antigen presentation in the skin are dendritic cells (DC) and Langerhans cells (LC). LC have a special ontogeny, as they originally are fetal liver- and yolk sac-derived macrophages that are seeded in the epidermis during embryonic development but can also be replenished from blood-derived myeloid cells¹⁵⁻¹⁷. Still, their functions are DC-like. LC can extrude dendrites beyond keratinocyte tight junctions to take up external antigens without compromising the barrier function¹⁸, thereby constantly surveilling the environment.

DC reside in the dermis and comprise several subsets (reviewed by Collin & Bigley¹⁹). As a simplistic overview, myeloid conventional (c)DC can be divided into cDC1, which are efficient in cross-presentation of antigens via MHC Class I to CD8⁺ T cells, while cDC2 present antigens to CD4⁺ T cells via MHC Class II. Plasmacytoid DC also cross-present antigens but are usually only found in the skin in inflammatory settings. In this context, they produce high amounts of type I IFN and thereby contribute to pathogenesis, for example in psoriasis or cutaneous lupus^{20,21}. Additionally, monocyte-derived DC can differentiate in the skin from blood monocytes. Even though these classifications can be useful for comparative analysis and recognition of broad patterns, DC subsets and functions are more heterogeneous and highly context dependent.

Both DC and LC can migrate to skin-draining lymph nodes upon activation and activate and polarize T cells and B cells, thereby instructing the nature and magnitude of the adaptive immune response²². In secondary immune responses, these APC can also locally present antigen to memory T cells that reside in the skin²³, mediating fast and localized recall responses and maintaining skin homeostasis.

Structural cells – non-trivial contributors to skin immunity

Skin as a barrier organ is constantly exposed to environmental threats, hence immune functions are also carried out by non-hematopoietic tissue cells. In addition to mechanical, physical and chemical defense mechanisms, structural cells of the skin are equipped with a wide range of pattern recognition receptors (PRR) to detect foreign and harmful agents. Both keratinocytes in the epidermis and fibroblasts in the dermis express PRR and upon activation initiate type I interferon responses and release pro-inflammatory cytokines, such as IL-1 α , IL-1 β , IL-6 or TNF- α and chemokines, such as CCL20, CCL27, CXCL9 or CXCL10 to recruit immune cells (reviewed by Klicznik et al²⁴, and Cavagnero & Gallo²⁵). At the same time structural cells can control excessive inflammation by releasing immunomodulatory cytokines, for example TGF- β , limiting IFN- γ responses, promoting wound healing, and supporting regulatory T cell function²⁶⁻²⁸. Overall, the context- and tissue-specific immune functions of structural cells are incompletely understood. A recent mouse study profiling the transcriptional and epigenetic landscape of different structural cells across organs, including epithelium, endothelium and fibroblasts, revealed organ-specific immune features and an epigenetic profile with open chromatin regions enriched for immune genes, but not expressed at steady state²⁹. This so-called unrealized epigenetic immune potential is readily activated in response to viral infection and reflects the organ-specific, rather than cell type specific patterns observed at steady-state²⁹.

Another open question is whether structural cells have antigen presentation functions. While it is established that structural cells can present antigens to CD8⁺ T cells via MHC Class I, there is increasing evidence that in inflammatory conditions, structural cells might be able to

present antigen to CD4⁺ T cells via MHC Class II, a feature classically attributed to professional APC only. Keratinocytes, fibroblasts and endothelial cells in the skin upregulate HLA-DR and co-receptor molecules in response to pro-inflammatory cytokines³⁰⁻³². *In vitro* assays suggest that these stimulated cells can elicit antigen-specific T cell activation³³. *In vivo* models indicate that epithelial cells in lung and gut act as non-professional APC for CD4⁺ T cells^{34,35}. However, functional evidence for class II presentation by human skin structural cells is so far missing.

Besides potential antigen presentation, structural cells communicate with immune cells through a plethora of contact dependent and secreted signals and provide crucial niche signals for the maintenance of immune cells. Especially tissue-resident cells that permanently reside in the skin depend on structural cells providing adhesion cues, growth factors and cytokines for their survival³⁶⁻³⁸.

Adaptive immunity and tissue residency in the skin

Adaptive immune responses in the skin occur when memory cells are generated in response to a primary infection. In the skin, resident cells of the adaptive immune system are virtually only composed of T cells. B cells are rare in healthy human skin and B cell infiltration is associated with inflammatory diseases, such as Pemphigus vulgaris or cutaneous Lupus erythematosus³⁹⁻⁴¹. However, the number of T cells in healthy human skin doubles the amount of T cells that circulate in the blood, making it one of the largest T cell reservoirs of the body⁴². Skin contains CD4⁺ T cells with different polarizations and functions, such as CD4⁺ T helper (Th) cells, comprising Th1, Th2 and Th17 as well as regulatory T cells (Treg)¹. Among skin CD8⁺ T cells (Tc), there are cells with Tc1 and Tc17 polarization¹. Classical immunology postulates that type 1 responses (Th1, Tc1) confer cell-mediated immunity and occur upon intracellular infections with viruses or bacteria (reviewed by Anunziato et al⁴³). The cytokines IFN- γ and IL-2 as well as the transcription factor T-bet are type 1 hallmarks. Th2 responses are associated with allergy and helminth infection, are tightly entangled with humoral immune responses and involve GATA3, IL-4, IL-5 and IL-13 signaling⁴³. Type 17 responses (Tc17, Th17) dominate in barrier tissue immune responses to extracellular bacteria and fungi. ROR γ t is the master transcription factor of this lineage, and type 17 cells produce IL-17A, IL-17F, IL-21 and IL-22⁴³. Treg on the other hand are important to maintain homeostasis and self-tolerance, depend on transcription factor Foxp3 and produce immunoregulatory cytokines, such as IL-10 and TGF- β (reviewed by Boothby et al⁴⁴). Most of these T cells in the skin are tissue-resident and highly specialized for long-term tissue maintenance. The next chapter will describe the current state-of-the-art regarding TRM biology and functions in human skin in detail.

1.2. Tissue-resident memory T cells

The skin contains vast numbers of T cells exerting their immune functions *in situ*. For the longest time it was thought that memory T cells generated during infection migrate to the tissue to clear the initial immune trigger and then either die in the tissue or circulate back to the lymph system, patrolling lymph nodes to wait for a secondary antigen encounter. However, in the beginning of the 21st century researchers discovered that some T cells permanently reside in peripheral tissues and represent a self-sustained population which is independent from circulating T cells. These cells were subsequently termed tissue-resident memory T cells (TRM).

CD8⁺ TRM were initially described in murine adoptive transfer infection experiments in gut⁴⁵ and skin^{46,47}, characterized by the absence of classic T central memory (TCM) and T effector memory (TEM) markers, such as CCR7 and CD62L, and defined by a phenotypically distinct expression profile of the integrin CD103 and the activation marker CD69. Since then, TRM have been intensely studied due to their prevalence in virtually all human tissues and involvement in several diseases. This chapter will summarize the current knowledge in TRM biology, regarding their development and maintenance, their function in homeostasis and involvement in several pathologies with a focus on the skin. Furthermore, I will discuss commonly used model systems to study TRM and highlight the importance for human/humanized model systems to investigate TRM biology in the skin.

1.2.1. TRM development and maintenance

TRM are memory T cells that are generated in peripheral tissues during primary antigen exposure from antigen-specific infiltrating T cells. During their generation, they must undergo transcriptional reprogramming, allowing them to adapt to a resident phenotype and survive in the tissue environment. The mechanisms underlying this transition have been mostly studied in murine CD8⁺ TRM in different organs and infection models, some of which could be confirmed in human cells and apply to the CD4⁺ TRM compartment. However, we are far from understanding how the full process of tissue adaptation is orchestrated in specific T cell populations in any given tissue. The most important pathways regulating residency, retention and recirculation of skin TRM are summarized in Figure 2.

TRM development, maintenance and retrograde migration

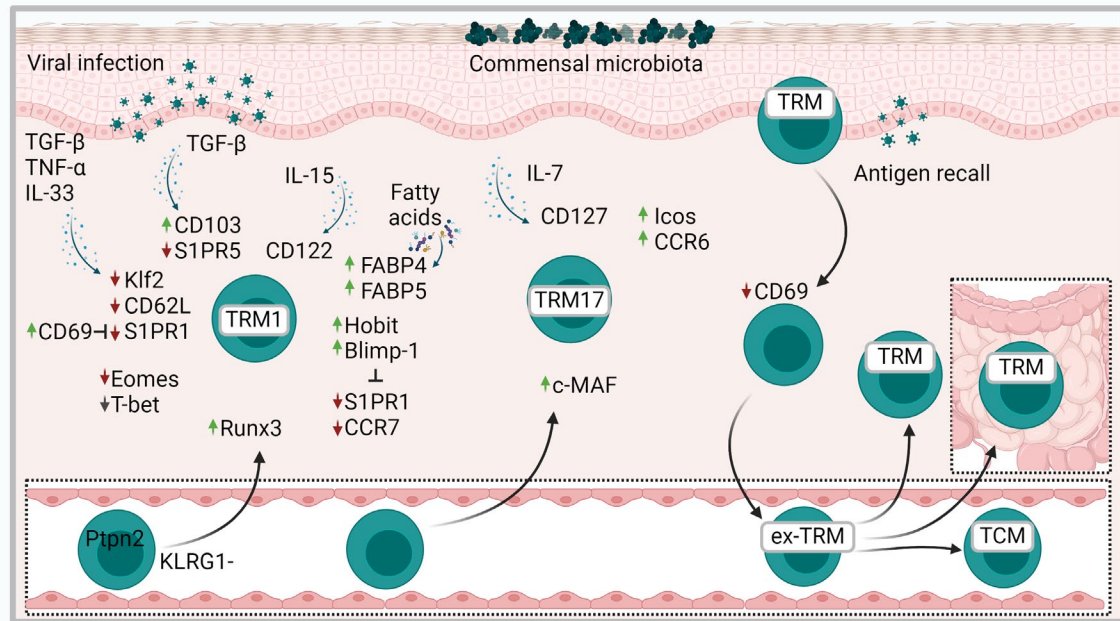


Figure 2 – Transcriptional, metabolic and migratory properties of skin TRM

The left part of the graph depicts an overview of transcriptional mechanisms regulating the generation of TRM1, generated in response to viral infection, and TRM17, generated during exposure to commensal bacteria. Red arrows indicate requirement for downregulation of the respective genes/proteins, green arrows indicate sustained expression or upregulation of the indicated molecules. Shown cytokines either down-or upregulate certain gene sets, or signal to their respective ligands, mediating cell survival. The right part of the graph illustrates ex-TRM fates after retrograde migration from the tissue during antigen recall. Mechanisms depicted here apply to mouse CD8⁺ TRM and expression patterns of most genes have been confirmed in human TRM.

Transcriptional regulation of the residency program

Early reports demonstrated that murine CD8⁺ TRM arise from KLRG1⁻ precursors during primary infection and upregulate the TRM marker CD103 in response to tissue-derived TGF- β ⁴⁸. Deficiency in *Ptpn2* expression impairs skin seeding of KLRG1⁻ TRM precursors⁴⁹. In mouse skin, expression of CD103 is required for long-term epidermal location of TRM⁴⁸. Human skin harbors large amounts of CD103⁻ CD4⁺ TRM in the dermis, as well as both CD4⁺ CD103⁺ and CD8⁺ CD103⁺ in the epidermis⁵⁰. This contrasts with mice, where epidermal TRM are exclusively CD8⁺, whereas dermal CD4⁺ T cells in naïve skin are mostly recirculating and, after immune challenge, only around 50% become resident⁵¹.

One requirement for TRM formation is the downregulation of transcription factor KLF2 and its downstream targets, CD62L and the sphingosine-1 phosphate receptor S1PR1⁵². S1PR1 allows T cells to recirculate following S1P gradients, which are high in blood and lymph. The TRM marker CD69 competitively binds to S1PR1, further preventing T cell tissue egress^{53,54}.

KLF2 downregulation can be induced by tissue cytokines, such as TNF- α , TGF- β or IL-33⁵². Recently, another S1P receptor, S1PR5, was implicated in TRM formation. Evrard et al could show that T-bet and ZEB2 signaling induces S1PR5 after antigen experience and that TGF- β -mediated S1PR5 downregulation is necessary to properly form CD8⁺ TRM upon skin infection⁵⁵. Further tissue-derived signals such as IL-15 and IL-7 are needed for the maintenance and survival of TRM^{48,56}.

CD8⁺ TRM development further depends on the coordinated downregulation of T-box transcription factors Eomes and T-bet⁵⁷, although residual T-bet signaling is necessary to maintain expression of the IL-15 receptor beta chain CD122⁵⁷. Another important transcription factor module for murine TRM development is Hobit-Blimp1⁵⁸. Whereas Hobit is essential for TRM development across tissues, its homologue Blimp1 synergizes with Hobit to promote TRM, by repression of S1PR1 and CCR7, which are molecules enabling recirculation^{58,59}. Studies in TRM reporter/deleter mice identified Hobit⁺ TRM precursors with downregulated Eomes expression among effector cells in the blood, hinting at early TRM fate imprinting before tissue entry⁶⁰. This early TRM fate commitment before skin entry was also observed in lineage-tracing experiments⁶¹, and intestinal TRM require priming through retinoic acid signaling in the mesenteric lymph node for development⁶². Of note, the human homologues of *Hobit* (*ZNF683*) and *Blimp1* (*PRDM1*) are expressed in human TRM, although at lower levels compared to murine TRM⁶³⁻⁶⁵.

Additionally, Runx family transcription factors control TRM development. CD8⁺ TRM undergo Runx3-driven transcriptional changes that allow adaptation to a TGF- β -driven residency program⁶⁶. This is absent in CD4⁺ TRM, which in turn require Runx1 for their development, although they express low levels of Runx3⁶⁶. Of note, this is one of few studies reporting specific requirements for CD4⁺ TRM, including two other studies reporting dependency on IL-7 in the lung of mice after bacterial infection⁶⁷ and CCR5 expression on CD4⁺ TRM in human mucosa⁶⁸.

Concerning RUNT family transcription factors in human TRM, RUNX2 and RUNX3 promote a distinct subset of cytotoxic CD8⁺ TRM that express CD49a⁶⁹. This specific TRM subset was first described by Cheuk et al in 2017, alongside a CD49a⁻ IL-17-producing subset⁷⁰. The discovery of these specialized subsets with distinct phenotypes and functions opened a new field in TRM research, aiming to uncover the plasticity of TRM and understanding the mechanisms that orchestrate phenotypic and functional variety. The next section will summarize current findings regarding this topic.

TRM heterogeneity and subset-specific requirements

The existence of distinct CD49a⁺ and CD49a⁻ TRM with different functional properties was among the first to describe TRM subset heterogeneity⁷⁰. TRM compartment heterogeneity was since then fortified with the advancing use of single-cell RNA sequencing technologies,

describing unexpected phenotypic plasticity within TRM cells in several tissues⁷¹⁻⁷⁴. Kurd et al found CD8⁺ TRM with diverse functional properties at progressive differentiation stages in response to viral infection⁷¹. Milner et al described effector-like Id3^{lo}Blimp1^{hi} TRM and memory-like Id3^{hi}Blimp1^{lo} TRM, which accumulate at distinct timepoints after infection⁷². In human intestinal transplant patients, two populations of CD103⁻ and CD103⁺ recipient-derived CD8⁺ TRM form in the gut, characterized by cytotoxicity and polyfunctional cytokine expression, respectively⁷³. Inflamed psoriatic and rheumatoid arthritis joints harbor distinct subsets of CD8⁺ TRM⁷⁴, hinting at the importance of TRM in mediating tissue inflammation in disease-specific ways. In infants and children, heterogeneity within the TRM compartment also reflects human development and maturation⁷⁵.

In 2023, Park et al provided the first evidence for distinct developmental trajectories of CD8⁺ TRM subsets in murine skin⁷⁶. Using different infection models, parabiosis experiments and transcription factor knockout systems, they established that type 1-polarized TRM (TRM1) and type-17 polarized TRM (TRM17) are generated and maintained via different signaling axes in the skin⁷⁶. CD49a⁺ TRM1 (which resemble the human CD49a⁺ cells described by Cheuk et al⁷⁰ and Zitti et al⁶⁹) primarily produce IFN- γ and develop along the previously described route depending on Hobit, IL-15 and residual T-bet expression upon herpes simplex virus (HSV) infection. CCR6⁺ TRM17 on the other hand, establish after *Staphylococcus epidermidis* association, are IL-17A⁺ and ICOS⁺ and require c-Maf and IL-7 for their development and survival. In this study, CD8⁺ c-Maf⁺ CD127⁺ T cells were also detected in human skin⁷⁶, and CD49a⁻ TRM described by Zitti et al have a profile matching those of TRM17⁷⁰, suggesting a similar dichotomy among human CD8⁺ TRM. Concerning human CD4⁺ TRM, few papers describe type 1 and type 17 specific TRM^{77,78} and proof for distinct developmental requirements for both CD4⁺ and CD8⁺ TRM subsets is lacking in humans.

In 2017, Kumar et al described the first core transcriptional signature for human TRM cells across multiple tissues, which included upregulation of molecules CD103, CD69, CD49a, CRTAM, PD-1, CXCR6, DUSP6 and CD101 (on CD8⁺ TRM), with simultaneous downregulation of KLF2, CD62L, S1PR1, S1PR5 and CXCR3⁷⁹. Since then, single-cell RNA sequencing of diverse human tissues have generated tissue-specific TRM transcriptome signatures which will be useful in the future to understand organ-specific TRM regulation and tissue adaptation mechanisms^{65,80}. Skin-specific TRM genes include *CXCR4*, *CCR10*, *GATA3* and *BCL6*, while other genes are shared by TRM across organs (*AHR*, *CXCR6*, *VIM*, *TNFSF9*)⁶⁵.

Metabolic tissue adaptation of TRM

Given that TRM are long-lived and inhabit tissue niches that are metabolically confined and distinct from the circulation, it is not surprising that they acquire special metabolic features. Specifically, skin TRM display increased lipid uptake, use of fatty acid oxidation and concurrent

upregulation of fatty acid binding proteins (FABP)⁸¹. While skin TRM mainly depend on FABP4 and FABP5 expression⁸¹, TRM in other organs express a different FABP pattern⁸². These expression patterns are tissue-specific rather than antigen-trigger dependent⁸². This was demonstrated in mice, where TRM in skin, liver and gut upregulate the same protein in an organ-specific manner across different infection systems, and organ-specific profiles are induced in response to systemically disseminating lymphocytic choriomeningitis (LCMV) infection⁸². Pathways that are activated in TRM response to fatty acids have so far only been studied in tumor resident CD8⁺ T cells⁸³, where TRM upregulated the transcription factor SCML4 via mTor-IRF4-PRDM1 signaling, thereby increasing anti-tumor activity. T cells display enhanced survival and effector capacity after fatty acid exposure and SCML4 upregulation, which the authors link to epigenetic reprogramming via HBO1-BRPF2-ING4⁸³. Besides FABP expression, TRM undergo further metabolic adaptation. *In vivo* CRISPR knockout screens in LCMV specific T cells targeting metabolic genes, combined with metabolomics in mice, uncovered the mevalonate-cholesterol synthesis pathway as positive regulators of small intestine (SI) CD8⁺ TRM⁸⁴. Follow-up experiments demonstrated that knockout of SREBP2, a master transcription factor regulating mevalonate-cholesterol pathway enzymes, impairs SI TRM generation. This is due to reduced formation of non-steroidal products, such as co-enzyme Q, which increase mitochondrial respiration in CD8⁺ SI TRM⁸⁴. In homeostatic conditions, resident CD8⁺ gut TRM reduce their mitochondrial content after sensing of prostaglandin E2, which induces mitochondria depolarization and subsequent autophagy-mediated clearance, which could be a mechanism to restrain the accumulation of T cells in the gut⁸⁵.

Another metabolic hub recently implicated in TRM regulation is polyamine metabolism. Polyamines play crucial roles in T cell activation and proliferation, as well as hypusination. In human and murine CD8⁺ T cells the glutamine/polyamine/hypusine axis-controls expression of core TRM marker CD69 and blocking the polyamine synthesis rate limiting enzyme ornithine decarboxylase significantly increases the generation of TRM in the bone marrow⁸⁶.

Overall, the different metabolic pathways that are engaged by TRM in different tissues and contexts, such as homeostasis, infection or cancer remain poorly understood. Discerning how TRM tailor their metabolism to ensure optimal immune responses will improve our basic understanding of immunosurveillance and allow the development of targeted interventions in cases where these optimal responses are disturbed.

TRM migration and recirculation

In the years after their initial discovery, TRM were viewed as terminally differentiated sessile cells that patrol a confined space in their tissue microenvironment. The concept of inside-out immunity postulated that TRM are at the endpoint of a developmental trajectory from naïve cells being primed in lymph nodes, becoming activated, homing to peripheral tissues and

finally developing into TRM⁸⁷. However, with advancing models to study TRM, it became clear that heterogeneity within the TRM pool allows some TRM cells to exit the tissue after acquisition of a canonical TRM phenotype. Initial work using photoconversion of Kaede-expressing T cells in the skin demonstrated that non-lymphoid tissue T cells can generate progeny in SLO upon antigen recall⁸⁸. Further studies led to a proof-of-concept that TRM can undergo trans-differentiation during so-called retrograde migration, wherein previously formed TRM re-enter the circulating T cell pool and home to other organs⁸⁹⁻⁹¹. Based on this, researchers put forward the theory of outside-in immunity, which would allow peripheral memory in the form of TRM to spread across large distances, conferring protection at other tissue sites upon secondary antigen exposure⁸⁹.

Parabiosis studies in mice allowed to track these circulating ex-TRM and demonstrated that tissue exit and trans-differentiation into circulating TCM-like cells is recall induced^{87,89}. Retrograde migration occurs from various tissues, including gut, female reproductive tract, and skin, suggesting that this is a shared feature of TRM across organs. However, ex-TRM keep the propensity to home back to the tissue of origin, indicating a certain degree of epigenetic imprinting⁸⁹. Importantly, elimination of primary TRM in infection models not only diminishes local secondary TRM formation and immune responses but also affects TEM in the spleen⁹⁰. One study in mice even suggests that after a primary infection, up to 30% of all memory T cells in the blood are ex-TRM⁹². The authors propose that continuous expansion of resident immune cells in peripheral tissues is necessary to accommodate increasing immune cell numbers with increasing microbial experience as the organism ages. In this study eventually up to 25% of cells in visceral organs were leukocytes, and these cells served as a reservoir for blood memory cells⁹². Consequently, outside-in immune responses can critically shape systemic immune memory.

In humans, retrograde migration occurs in skin CD4⁺ TRM, although at steady state rather than secondary infection⁹¹. These skin-emigrated cells can be found at very low percentages among blood memory T cells with a distinct phenotype of skin homing marker CLA and residency-marker CD103 co-expression, allowing them to re-enter distant skin sites⁹¹. One aim of this thesis was to study this specific subset in humans in the context of hematopoietic stem cell transplantation and graft-versus-host disease, which will be described in detail later. Generally, studying circulating T cells which partially resemble their tissue counterparts might be an exciting opportunity to study tissue-specific immune responses without the need for invasive biopsies in humans. Furthermore, once better characterized these cells could serve as blood biomarkers for inflammatory processes or infections in internal tissues. It is therefore crucial to study the mechanisms that mediate outside-in responses in detail and determine which tissue-typic properties are retained during retrograde migration and trans-differentiation.

1.2.2. Skin TRM in infection, inflammation and cancer

Skin TRM are major regulators of infectious and inflammatory skin diseases as well as skin cancer. Antigen-specific TRM are established during primary skin infection and due to their longevity and permanent presence in the skin they are crucial to provide early protective responses to secondary exposures. However, TRM also have the potential to elicit persistent and recurring inflammation when aberrantly activated. In the following section, I will describe the main roles of TRM in mediating protection from infection and tumor control, as well as their known pathogenic functions in several autoimmune and chronic inflammatory diseases.

Infectious disease control by TRM

Arguably, the most important role of TRM is to protect tissues from recurrent infections. Multiple mouse models for viral and bacterial skin infections have demonstrated their important role in mediating rapid and efficient recall responses. Gebhardt et al and Jiang et al were among the first to identify TRM and showed that in HSV and vaccinia virus skin infection, these skin resident T cells control re-infections with the same pathogen^{46,47}. TRM survive in the absence of persistent antigen⁹³ and even protect against unrelated viruses in an antigen-agnostic manner⁹⁴. Similar observations were made in diverse viral infection models in many different organs, for example LCMV infection in brain, kidney or intestine^{95,96}, or Influenza virus and respiratory syncytial virus infection in the lung⁹⁷⁻¹⁰⁰. In humans, antigen-specific TRM to Hepatitis B virus reside in the liver^{101,102}, skin-homing dengue virus-specific T cells are generated in natural infection¹⁰³ and the COVID-19 pandemic uncovered the generation of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2)-specific TRM in lung and nasal mucosa^{104,105}.

TRM are equally important to protect from bacterial and fungal infections. For instance, CD4+ TRM protect from *Candida albicans* and *Leishmania major* infection in the skin^{106,107} and *Klebsiella pneumonia*, *Streptococcus pneumoniae* or *Bordetella pertussis* in the lung^{67,108-110}. Of note, vector-borne pathogen transmission can be facilitated by vector-transmitted factors that suppress skin TRM, as in the case of tick-transmitted *Borellia burgdorferii*¹¹¹. On the other hand, *Staphylococcus aureus*-specific TRM protect the skin from persistent bacterial colonization, but also worsen atopic dermatitis (AD)-like symptoms in mice¹¹². These examples demonstrate the need for stringent regulation of TRM to ensure optimal protection while preventing detrimental effects, causing profound pathogenesis when they are dysregulated. The following section describes inflammatory skin diseases in which aberrant TRM responses play a crucial role in driving disease pathology.

TRM as mediators of autoimmunity and inflammatory skin disease

Psoriasis and AD represent the most common chronic inflammatory skin diseases, affecting millions of people worldwide^{113,114}. In both, T cells are implicated as major producers of canonical cytokines that mediate tissue damage^{56,115,116}. In psoriasis, IL-23-dependent TRM

produce IL-17A, IL-22 and TNF- α , which activates keratinocytes for hyperproliferation, and recruits and activates immune cells that amplify the inflammatory loop^{56,117,118}. For the treatment of psoriasis, there are several biologics on the market, targeting signaling of these cytokines, such as secukinumab (IL-17A signaling), ustekinumab and guselkumab (IL-23 signaling), or infliximab and etanercept (TNF- α signaling)¹¹⁹⁻¹²².

AD is classically characterized by increased expression of type 2 cytokines, such as IL-13 and IL-4, which can impair the skin barrier function, cause itch and induce fibrosis¹²³⁻¹²⁶. Although direct evidence for IL-4 producing TRM in AD patients is missing, IL-13 producing persistent TRM exist in human skin¹¹⁵. Dual blockade of IL-4 and IL-13 signaling by dupilumab is an effective treatment for AD patients^{127,128}, and is gaining attention for treatment of other type 2-mediated inflammatory diseases, such as prurigo nodularis¹²⁹. Also, the specific blockade of IL-13 alone is effective in moderate-to-severe AD patients¹³⁰.

On the other hand, TRM1 with high cytotoxicity and IFN- γ production have been implicated in autoimmune skin diseases. In vitiligo, cytotoxic CD8+ TRM1 are responsible for the targeted killing of melanocytes¹³¹, and blocking TRM1 survival factor IL-15 rescues the phenotype in mice¹³². Polymorphic light eruption lesions contain cytotoxic TRM and IL-15-producing macrophages¹³³. Similarly, IL-15-dependent cytotoxic T cells mediate hair loss in alopecia areata models¹³⁴, and in patients scalp TRM are significantly enriched¹³⁵.

Furthermore, a range of hypersensitivity responses is mediated by TRM, for example fixed drug eruption, toxic epidermal necrolysis or allergic contact dermatitis¹³⁶⁻¹³⁸. Lastly, TRM have an unclear role in the pathogenesis of aGVHD^{139,140}. An in-depth characterization of TRM phenotypes in aGVHD was one aim of this thesis and will be described in detail later.

In all these settings, TRM mediate local inflammation within lesions, but often the diseases disseminate over time. Whether pathogenic TRM are independently generated at different disease sites, or whether recirculating TRM subpopulations spread the pathology, is not clear yet and might be a disease-specific process.

TRM-mediated cancer surveillance and anti-tumor functions

Since TRM are specialized to recognize foreign antigens in peripheral tissues, they are also suited to protect from solid tumors. In the skin, melanoma is the tumor with the highest mortality rate (reviewed by Davis et al¹⁴¹). In a mouse model of autoimmune vitiligo, induced by melanoma challenge and subsequent tumor excision, melanocyte-specific CD8+ TRM persist¹⁴². These mice are protected from melanoma re-challenge¹⁴². A different group showed that melanoma-specific TRM generated before melanoma inoculation mediate protection¹⁴³. A subset of naïve mice injected with melanoma cells was protected from developing macroscopic tumor lesions by generation CD8+ TRM, controlling tumor outgrowth¹⁴³. These tumor-infiltrating CD8+ TRM depend on Runx3, and Runx3-deficient T cells fail to accumulate in tumors and lead to greater tumor outgrowth and mortality in mice⁹⁶.

TRM with the same clonotypes as tumor-infiltrating T cells persist for years in human skin and maintain durable memory functions¹⁴⁴. Melanoma-specific TRM can also be found in tumor-draining lymph nodes in both mice and humans, and expression of a TRM signature in these lymph nodes correlates with survival¹⁴⁵. Importantly, increased numbers of tumor-resident TRM in immunotherapy-naïve patients correlates with improved survival in anti-PD-1 immunotherapy¹⁴⁶. Further compelling evidence for the critical anti-tumor functions of TRM in human skin comes from studies investigating skin immunity of people living with human Immunodeficiency virus (HIV). Our lab could show that patients starting antiretroviral therapy late after primary infection irreversibly lose CXCR3+ TRM in the skin¹⁴⁷. Importantly these patients have a dramatically increased risk of developing human papilloma virus-related skin cancer¹⁴⁷.

In the case of mycosis fungoides, a form of cutaneous T cell lymphoma, TRM themselves are malignant cancer cells¹⁴⁸. So far, it is not well understood how the disease develops, but it is composed of heterologous T cell populations, with pronounced expansion of a top clone that drives the disease, alongside benign T cell infiltrates¹⁴⁸⁻¹⁵⁰. Therapeutic strategies include depletion of CCR4-expressing T cells via antibody-mediated cell-dependent cytotoxicity, but tumors often become drug resistant because cells lose CCR4 expression¹⁵¹.

The studies summarized in this chapter demonstrate that current therapeutic interventions affecting TRM are mostly targeting the effector cytokines they produce, but fail to eliminate the pathogenic cells themselves, often resulting in relapse. The previous chapter introduced developmentally distinct TRM17 expressing ICOS. Of note, TRM17 can be selectively depleted by ICOS blockade, without affecting the TRM1 compartment⁷⁶. These findings are highly relevant for clinical approaches aiming to eliminate pathogenic TRM subsets specifically, or to target only affected tissues. However, we just begin to understand the diverse functions and phenotypes within the TRM compartment, especially in humans. Going forward, we require reliable markers and signatures that delineate pathology-mediating TRM subsets, so we can devise strategies to selectively manipulate them. To define such markers, we need physiologically and pathologically relevant models that allow us to make meaningful and translatable conclusions. In the next chapter I will describe the most important mouse models, and commonly used human model systems to investigate skin TRM biology.

1.2.3. Models to study skin TRM

Most of our knowledge about TRM biology comes from mouse studies, which are instrumental to uncover mechanisms of residency and functional properties of TRM. Furthermore, they allow characterization of organ-specific features, as different tissues can be readily harvested from the same animal. However, not all features of murine TRM biology are translatable to humans, and some diseases lack adequate pre-clinical models that replicate diverse aspects of pathophysiology. This is why complementary studies on human samples are essential. In this chapter, I will briefly summarize the most important pre-clinical models and systems to study TRM in mice and humans.

Adoptive transfer, parabiosis and transplantation as the gold standard in pre-clinical models

One dilemma of studying TRM in tissues is that CD69 and CD103 are imperfect markers to identify them^{48,152}. CD69 can also be expressed on recently activated effector cells and CD103 is usually only expressed on a subset of cells that reside closer to the epidermis⁴⁸. While using these markers as a proxy for TRM is highly practical and sufficient for many questions, especially in combination with markers of circulating and effector cells, we likely underestimate TRM that do not express them¹⁵². Additionally, they do not allow conclusions on the history and fate of a given cell at the specific time the sample is analyzed. For this purpose, researchers developed sophisticated mouse models that allow lineage tracing of T cells, addressing questions of TRM development, longevity and migration properties.

Photoconversion of Kaede-expressing immune cells in lymph nodes, which subsequently display a different fluorescent label, demonstrated establishment of resident immune populations¹⁵³. However, this approach also labels cells that are in tissue vasculature at the time of conversion, and the fluorescent signal decreases over the course of several days and with cell proliferation¹⁵². Another approach is intravascular antibody labeling, where recirculating blood cells are labeled while cells residing in the parenchyma are spared. However, this approach is only useful to determine anatomic location, not residence per se^{154,155}.

Definite proof of non-recirculating resident cells came from parabiosis experiments. For this, the circulation of two congenic mice is combined so that all recirculating immune cells of the two parabionts equilibrate, while resident populations largely remain unique to the host mouse^{47,100,152}. These experiments led to important discoveries in the TRM field and are still used by groups mainly in the United States^{92,98,156}. However, there are ethical concerns and, in many European countries these experiments usually do not get approved by local authorities¹⁵⁷.

Genetic reporter mice are a good alternative to track at least certain TRM populations. For instance, Hobit fate-mapping revealed the existence of Hobit⁺ TRM precursors, which suppress expression of tissue-exit molecules early on^{59,60}, or retrograde migration of Hobit⁺

TRM to lymph nodes⁹⁰. Similarly, knock-out models of several transcription factors proved their pivotal role in TRM function^{58,76,96}. Other approaches include the transplantation of solid tissues, which contain resident TRM populations of the donor mouse^{46,89,107,142,158}, as well as adoptive transfer of congenic and/or genetically modified T cells that can be traced in the host animal^{57,60,66,96}.

Although pre-clinical mouse models are crucial to study TRM biology *in vivo*, and many findings can be translated to humans, there are species-specificities. Especially regarding skin, the anatomy and immune composition of murine and human skin are quite different (reviewed in Pasparakis et al¹⁵⁹). Humans have a thicker epidermis and dermis than mice. While mouse skin is dense with hair follicles, in human epidermis a larger proportion is interfollicular. There are special T cells in murine epidermis, so-called dendritic-epidermal T cells (DETC) expressing $\gamma\delta$ T cell receptor (TCR), a cell type which does not exist in human skin. In general, $\gamma\delta$ T cells carry out important functions in mouse skin that, in humans, are likely taken over by different T cell subsets¹⁶⁰, as they are sparse in healthy human skin¹⁶¹. Therefore, it is important to study human TRM biology in human tissues and devise good models that can inform on physiological processes.

Experimental approaches to study human TRM

Human skin is an easily accessible organ, and many researchers use it to study the biology of resident immune cells, as biopsies are readily available. TRM in these biopsies are usually identified by expression of CD69 and/or CD103, along with CD49a, CXCR6, CCR8, CCR4, CCR6 and others^{42,50,69,70,79,162}. Methodologically, tissues are usually phenotyped by immunofluorescence or immunohistochemistry of skin sections, or flow cytometry and other immunological readouts on digested skin single-cell suspensions^{50,69,70}. Usage of these methods has facilitated important discoveries, such as the depletion of CXCR3⁺ TRM in HIV-infected individuals¹⁴⁷, or the presence of CCR6⁺ TRM near CCL20⁺ keratinocytes in never-lesional skin of psoriatic patients¹¹⁷. However, these approaches only give a static picture of the tissue at a given time point and fail to represent dynamic processes. This can be partially circumvented by longitudinal sampling^{139,146,163}, but is usually limited to a few biopsies that can be obtained from individual patients, or entails non-matched samples across time-points, which can be highly heterogeneous.

The advance of single-cell RNA sequencing technologies enables in-depth transcriptional analysis of T cells. Recently, also paired analysis of mRNA and TCR profiles of individual cells is possible, allowing to connect phenotypes and clonotypes across different tissues in humans to identify novel markers and pathways involved in TRM biology⁶⁵.

There are additional methods to mimic dynamic processes of human TRM *ex vivo*. Explant systems of whole human skin allow live-cell imaging of TRM *in situ*¹⁶⁴ or profiling of immune cell responses to an experimental tick bite by injection of tick salivary gland extract and

*Borrelia burgdorferi*¹¹¹. Blood T cells can adapt TRM-like features in 2D co-culture systems settings with keratinocytes or fibroblasts¹⁶⁵, and mimic inflammatory disease phenotypes in 3D skin equivalents¹⁶⁶. Still, most skin equivalent models lack organotypic features, such as the vasculature or hair follicles. Although vascularized and hair-bearing skin organoids have been developed from pluripotent or embryonic stem cells, those lack a functional immune compartment^{167,168}. Furthermore, while these organoids have great potential to advance our understanding of many physiological and pathological processes occurring in human skin, they are laborious and cannot be easily set-up and reproduced across different laboratories. At the moment, combining single-cell RNA sequencing technologies with phenotypic analysis and co-culture assays is a multi-layered, feasible and informative approach to study human TRM. Especially combining single cell RNA sequencing with spatial profiling can be powerful to identify cellular processes in tissues¹⁶⁹⁻¹⁷¹.

In our lab, we have made important discoveries about the longevity of TRM by studying them in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT). While resident and recirculating T cell can be distinguished in mouse models of parabiosis or adoptive T cell transfer with congenic markers, this cannot be replicated in humans. However, transplantation is a unique clinical setting where two cellular genotypes exist within one individual and can be used to some degree to track specific immune cell fates in humans. This was harnessed by many researchers to uncover the migratory behaviors of certain immune cells as well as the persistence and longevity of human TRM in different organs^{73,77,172,173}. Methodologically, host and donor cells can be distinguished by antibody-staining for discordant HLA-types^{73,172}, or fluorescence in-situ hybridization (FISH) specific for X and Y chromosomes, in the case of sex-mismatched transplantations^{77,139,173}. In single-cell RNA sequencing-based approaches, single-nucleotide polymorphisms (SNP) that are specific to host or donor cells can be used to distinguish genotypes¹³⁹.

For the projects in this thesis, we have made use of these methods to investigate migratory behavior and heterogeneric functions of skin TRM in aGVHD as classical T cell-mediated inflammatory disease and assess the reconstitution dynamics of TRM in HSCT in sequentially taken skin samples. The next chapter features an overview of the clinical background on this unique patient cohort and a detailed description of the specific role of T cells in the pathophysiology of aGVHD.

1.3. Human skin TRM in hematopoietic stem cell transplantation and graft-versus-host disease

HSCT is often the only curative treatment for patients suffering from various types of hematopoietic disorders, such as acute myeloid leukemia, myelodysplastic syndrome, or acute lymphoblastic leukemia, as well as patients with immunodeficiencies or blood production disorders, such as hemophagocytic disorders or aplastic anemia¹⁷⁴. These patients usually undergo myeloablative conditioning to ablate the host hematopoietic system and subsequently receive peripheral blood-mobilized or bone marrow-derived stem cells. While many patients are eligible for autologous transplantation, a significant proportion undergoes allogeneic transplantation. In Austria, more than 600 people per year receive a HSCT and around 25% of those are transplanted with allogeneic stem cells from a related or unrelated donor (Austria, Transplant annual report 2019¹⁷⁵). However, these patients are at high risk of developing GVHD, a multi-systems inflammatory disease with a high mortality rate, making it the main life-threatening complication after HSCT.

Clinically, GVHD can be divided into two major disease phenotypes – acute (a) and chronic (c)GVHD. Although the clinical manifestations and involved cell types differ, the same underlying immunological principle is thought to be the driver: donor immune cell activation (mostly T but also B cells in cGVHD) by APC that present allogeneic peptides (reviewed by Zeiser & Blazar^{176,177}). These activated cells then induce an inflammatory response, leading to organ damage. GVHD is usually treated with immunosuppressants (steroids, JAK/STAT inhibitors), but mortality remains high in these patients, calling for better treatment options. Skin, alongside gut and liver, is the most commonly affected target organ in aGVHD and cGVHD and often the first site of disease manifestation. Due to the important role of T cells in both skin immunosurveillance and GVHD pathogenesis, aGVHD is regarded a model disease to study pro-inflammatory T cell processes in the skin. In this thesis we investigated TRM functions in the context of cutaneous aGVHD. Therefore, the next chapters will summarize current knowledge about the pathophysiology of aGVHD and how T cells, and specifically TRM, are involved in these processes. Furthermore, I will discuss current reports about host and donor TRM chimerism following HSCT and outline how studying T cell responses in HSCT patient samples can inform on basic principles of TRM function and biology.

Concerning cGVHD, we summarized the pathophysiology mediated by different immune cell types, including T cells, in a published review on which I am first author. This review is included in the Appendix of this thesis (see 6.1.) and therefore mechanisms of cGVHD pathology will not be discussed further in the introduction.

1.3.1. Pathophysiology of acute GVHD

Acute GVHD is a T cell-driven inflammatory disease, mediated by donor T cells reacting against host antigens. It is thought to arise due to genetic disparities between host and donor. These genetic mismatches mainly lie within the human leukocyte antigen (HLA) locus and immune responses are mostly directed against host MHC molecules and minor histocompatibility antigens^{178,179}. Mismatches in certain HLA genes, such as MHC class I chain-related gene A (MICA), are associated with a higher risk for aGVHD¹⁸⁰. The degree of minor histocompatibility antigen mismatch is another risk factor that increases aGVHD incidence, although not all mismatches are equally immunogenic¹⁸¹. Additionally, other genetic and non-genetic factors might contribute to the disease, and the exact reason why aGVHD arises in some patients but not in others is unclear and cannot only be explained by matching of HLA genotypes¹⁸²⁻¹⁸⁴.

Importantly, higher degree of matching usually also decreases the graft-versus-leukemia (GVL) effect, which is required to kill residual leukemic cells and therefore puts the patients at greater risk for relapse. To date, reliable biomarkers that predict early onset of aGVHD are lacking. First-line treatment usually consists of high-dose glucocorticoids, but many patients become steroid-refractory, with aGVHD recurrence and poor long-term prognosis¹⁷⁶. Even though there have been major advancements in the prevention and treatment of aGVHD, such as the use of post-transplant cyclophosphamide in aGVHD prophylaxis^{185,186}, or the recent approval of the JAK inhibitor Ruxolitinib for treatment of steroid-refractory patients¹⁸⁷, there is a high rate of aGVHD relapse and aGVHD-related mortality. Therefore, there is a clinical need for better biomarkers to stratify patients, and improved preventive measures that allow minor mismatch transplantation to secure GVL while abrogating aGVHD development.

A hallmark of aGVHD is tissue damage through activated donor T cell responses. Initially, tissue damage caused by myeloablative conditioning creates a pro-inflammatory milieu with barrier breach and microbial dysbiosis that elicits APC activation and priming of alloreactive T cells in SLO¹⁷⁶. After allo-priming, T cells recognizing their cognate antigen in the target tissue, induce effector responses, including cytotoxic responses and production of pro-inflammatory mediators that recruit and activate other immune cells¹⁷⁶. Allo-priming can be mediated by both remaining host APC or donor APC, and in target tissues antigens may even be presented by non-hematopoietic cells via MHC Class II¹⁸⁸. The main phases of aGVHD pathology are illustrated in Figure 3. Researchers have tried to discern the underlying signaling molecules that are implicated in these processes in aGVHD. Most of our current knowledge comes from mouse models of allogeneic mismatched bone marrow transplantation (alloBMT), which can be combined with sophisticated cell tracking and knock-out models to study the role of individual cell types or genes. These studies have highlighted important differences and commonalities in pathomechanisms within tissues most commonly affected by GVHD (skin, gastrointestinal tract, and liver) and the associated secondary lymphoid structures and

revealed several important players in the priming and effector phase of aGVHD. This chapter will highlight recently identified molecular players that control T cell pathogenicity in aGVHD.

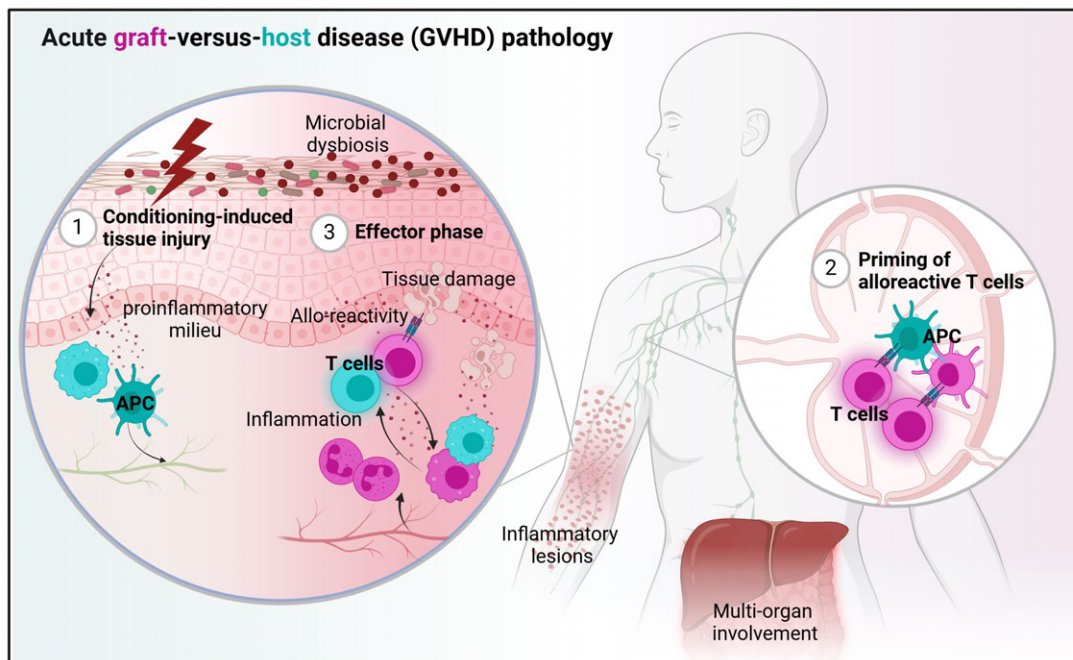


Figure 3 – T cells in acute GVHD pathology

Simplified schematic of acute GVHD pathophysiology in the skin, with initial tissue injury causing pro-inflammatory signaling and activation of APC, which then prime alloreactive T cells in secondary lymphoid organs. Activated allo-reactive T cells infiltrate target tissues and cause cytotoxicity and activation and recruitment of other immune cells to lesional skin, mediating extensive tissue damage and amplifying the inflammatory loop.

Allogeneic T cell priming in secondary lymphoid organs

In the initial phase of aGVHD development, patients experience tissue damage caused by radio-chemotherapy, leading to the release of pro-inflammatory mediators, APC activation, the promotion of allogeneic T cell priming and activation in SLO, which then infiltrate target tissues¹⁷⁶. At this stage, the alarmin IL-33 produced by fibroblastic reticular cells (FRC) in SLO was shown to promote the expansion of allogeneic T-bet-expressing Th1 cells by signaling via the IL-33 receptor ST2 on T cells¹⁸⁹. Similarly, FRC deliver activating Notch signals to T cells in SLO, and their inactivation prevents GVHD in these mice¹⁹⁰. Another study described a subset of CCL19+ FRC, expressing MHC Class II, which dampens GVHD during the effector phase by promoting the expansion of donor Treg¹⁹¹.

The developmental relationships of lymph node-primed and tissue effector cells are not yet fully understood. A recent study deploying single-cell RNA sequencing on allogeneic donor CD4+ T cell fates in mesenteric lymph nodes and gut during aGVHD exacerbation uncovered that within a single developmental trajectory cells acquire pathogenic or regulatory states¹⁹². Importantly, this analysis revealed a second trajectory of quiescent stem-like TCF-1^{hi} T cells, which home to the gut and give rise to new effector cells in a secondary recall response¹⁹².

These studies showcase that SLO instruct pathogenic tissue responses, and that targeted interventions at this stage could be effective to prevent excessive tissue damage and full blown aGVHD development. However, SLO-instructed effector programs are not irreversibly imprinted. Clonally related effector CD8⁺ T cells from skin or gut and their corresponding draining lymph nodes display highly divergent transcriptional profiles, indicating that these cells are reprogrammed within the target tissue, where the lymph node-imprinted programs are overwritten¹⁹³. For instance, in skin GVHD, Notch signals derived from Langerhans cells in the murine epidermis mediate a Notch-dependent T cell activation program and local pathogenicity upon epidermal entry¹⁹³.

Approaches to confine T cells in SLO and prevent their migration to target tissues were successful in pre-clinical models. Lipid mediators, like S1P or ceramide, regulate diverse immune cell functions such as migration and proliferation. Modulating the sphingosine-1-phosphate receptor S1PR1 with Fingolimod (FTY720) traps T cells in SLO and ameliorates GVHD in alloBMT models^{194,195}. Another study demonstrated a beneficial effect of S1P1 blockade by reduced macrophage recruitment into target tissues¹⁹⁶. Of note, FTY720 also impairs GVL, making its clinical application precarious¹⁹⁷. Inhibiting ceramide synthesis by deletion or therapeutic targeting of ceramide synthase 6 significantly ameliorates aGVHD, while preserving the GVL effect¹⁹⁸. This study reported reduced IFN- γ production in donor T cells and retention in SLO¹⁹⁸. Mechanistically, ceramide synthase 6 is required for optimal assembly and signaling through the TCR/CD3 complex and downstream activation of N-RAS and ERK signaling pathway especially in CD4⁺ T cells¹⁹⁸.

Collectively, these studies indicate that targeting alloreactive T cells in SLO might be a promising strategy for reducing allogeneic responses. However, T cells that still reach target tissues can be transcriptionally reprogrammed by tissue-derived signals, overwriting signatures acquired in SLO. Ideally, one would devise strategies that favor the expansion of regulatory cells, while abrogating the development of pathogenic or stem-cell like effectors. To address this, we need to carefully dissect the different T cell phenotypes that arise during aGVHD and assess mechanisms of their regulation.

T cell diversity in allogeneic immune responses

The priming of effector T cells in allogeneic immune responses generates diverse effector T cell subsets, which subsequently migrate into target tissues and exert their functions within those environments. These tissue infiltrates are composed differently in target organs of aGVHD. In the gut, many pathogenic T cells are polarized to a type 1 or type 17 profile, while in the skin type 1 and type 2 immune responses dominate¹⁹⁹.

While the detrimental effects of type 1 immune responses are well studied, the role of type 17 responses is still being investigated. Most studies support their pathogenic function in aGVHD, often in concert with type 1 responses²⁰⁰⁻²⁰³. A recent mouse study reported that transferred

Th17 cells promote GVHD by simultaneous production of IL-17A and IFN- γ , whereas IL-17A alone suppresses Th1 cells by downregulating IL-12 in donor macrophages, thereby alleviating aGVHD²⁰³. Complement proteins can regulate the allo-activation of T cells and modulate adaptive immune responses. Complement protein C3 deficient recipient mice have reduced GVHD with decreased numbers of Th1/Th17-polarized donor T cells²⁰⁴ and treatment with the C3-inhibitor Compstatin replicates this phenotype, with little effect on CD8+ T cells²⁰⁵. C3 deposits can be detected in human skin aGVHD biopsies²⁰⁵. However, functional evidence for Th1/Th17 polarization mediated by C3 in human skin is still missing.

IL-6 and IL-23 are established cytokines inducing Th17 polarization, and several recent studies implicated these cytokines or their receptors in disease pathogenesis. Mice with IL-6-deficient DC have less Th17 and lower expansion of allo-specific T cells after alloBMT²⁰⁶. Moreover, a distinct subset of Tc17 cells forms shortly after alloBMT, depends on IL-6 from host cells, and is highly inflammatory without significant GVL capacity²⁰⁷. Their deletion prevents lethal GVHD, but their lineage is unstable and over time they acquire a Tc1 phenotype, which is the reason that they cannot be detected without lineage tracing²⁰⁷. Ball et al described retinoic acid-responsive IL23R+ effector CD8+ T cells, which are strongly enriched at sites of tissue damage, as well as high IL-23 levels in patients with gastrointestinal (GI) aGVHD²⁰⁸. Bastian et al found the novel IL-12 cytokine-family member, IL-39, to upregulate IL23R in human T cells, and expression of the IL-23R subunit IL-23R α is required for aGVHD induction in their mouse model²⁰⁹. Mice receiving IL-23R α -mutant T cells also display lower levels of GM-CSF²⁰⁹, another important aGVHD cytokine. GM-CSF derived from both Th17/Tc17 positive and Th17/Tc17 negative cells promotes allo-antigen presentation by donor DC in the colon and lymph node²¹⁰. In a subset of pathogenic colonic CD4+ T cells, GM-CSF production is regulated by transcription factor Bhlhe40²¹¹. However, these Bhlhe40+ GM-CSF+ CD4+ T cells are independent from type 17-inducing cytokines IL-6 and IL-23, but rather showed high expression of transcription factor genes related to the Th1 or Th2 lineage (*Tbx21*, *Gata3*)²¹¹.

Within the epidermis, also keratinocytes can deliver pathogenic signals to T cells. In *in vitro* models, keratinocytes expressing CD58 and CD54 (ICAM-1) induce Th1 and Th17 differentiation in inflammatory conditions by co-stimulating their receptors CD2 and LFA-1, respectively²¹². Similarly, keratinocytes expressing CD166 and CD318 signaling to T cells via CD6 downregulates CCR7 and induces mTOR signaling, thereby promoting T cell activation and proliferation²¹³. Fibroblasts within the dermis upregulate allograft inflammatory factor-1 (AIF1) in response to tissue damage in sclerodermatous GVHD, which increases IL-6²¹⁴. As described above, IL-6 is an important pro-inflammatory mediator in aGVHD. However, little is known about the influence of fibroblasts on aGVHD pathogenesis. As non-hematopoietic cells can be targeted by alloreactive T cells during GVHD, even without expressing cognate allo-

antigens, and in response upregulate molecules like ICAM-1²¹⁵, it is likely that they play an important role in the retention of T cells in target tissues and thus in sustaining inflammation.

Context- and tissue-specific disease-regulatory mechanisms in GVHD

The pathology of aGVHD is complex and several studies highlight that individual pathways or components can have opposing effects depending on the presence of other cellular players. For instance, GVHD is promoted by Th17 transferred cells but IL-17A alone is protective²⁰³, and dermal macrophages are recruited after myeloablative, but not non-myeloablative conditioning²¹⁶.

Although we have gained important insights into disease pathology from animal models, these models tend to underestimate the complexity of cell type heterogeneity and co-dependency. Additionally, animal experiments are often influenced by housing conditions, such as the facility-specific microbiome, fail to consider the effect of immunosuppression used after clinical HSCT, or use transplantation models that are not clinically relevant. For example, few studies in mice consider potential differences between the commonly used full-mismatch models of alloBMT and MHC-matched transplantation. The latter would have more translational relevance, as patients mostly receive an HLA-matched or haplo-identical transplant. The impact of this was demonstrated by a recent study from Bader et al, revealing that STING knockout ameliorates GVHD in MHC-matched transplantation by reducing the activation of donor CD8+ T cells, the main effectors in this transplantation model²¹⁷. However, STING knockout also promotes the survival of MHC Class II+ APC, which increases the activation of CD4+ T cells. These are the main GVHD effector cells in mouse models of mismatched transplantation, thereby promoting disease in this setting²¹⁷. While these model-specific effects should be considered and discussed in future mouse studies, they also reflect the diverse parameters that can vary in clinical HSCT and may be the cause for different treatment responses in aGVHD patients.

Overall, the pathogenic processes promoting and maintaining alloreactivity of T cells in aGVHD are highly complex and might depend on disease stage, tissue and T cell subset in addition to clinical parameters. Although there are many promising molecules that can be effectively targeted in mouse models, such as C3 or ceramide 6 synthase, most of them have not been validated in humans. To develop effective and personalized treatment plans for patients, we need better models and studies in human/humanized systems that can account for effects of the conditioning regimen, transplantation scheme and specific aGVHD organ involvement. In the next chapter, I will describe why serial tissue sampling of patients undergoing HSCT represents a unique opportunity to not only understand aGVHD pathophysiology, but also study basic principles of T cell function in homeostasis and inflammation.

1.3.2. Role of TRM in HSCT and aGVHD

HSCT patients represent a unique opportunity to study T cell biology *in vivo*. Examining processes of immune reconstitution after radio-chemotherapy has led to important discoveries, such as early establishment of a stable circulating TCR repertoire after transplantation, that is significantly altered by cytomegalovirus reactivation²¹⁸. Tracing of retrovirally engineered T cells in the blood of patients receiving HSCT in clinical trials applying gene therapy revealed that T memory stem cells can persist for up to 12 years after infusion²¹⁹. Clonal tracking of CD8+ T cells in donor-recipient pairs uncovered that CD8+ TEM displaying long-term persistence in recipient blood can be isolated by specific surface markers from donor blood²²⁰. However, most of the research in pre-clinical models and human material has been focused on studying allogeneic T cell responses in the blood. The discovery of TRM has prompted investigations into TRM reconstitution after HSCT and into their role in aGVHD disease pathology, which I will summarize in this chapter.

Pathogenicity by local donor T cell responses in aGVHD target tissues

Skin, besides liver and gut, is the most common target organ in aGVHD. Of note, these are barrier organs that harbor vast amounts of TRM. Detection of donor-derived T cells in human skin as early as 14 days after HSCT revealed that they quickly acquire a TRM phenotype¹³⁹. In a non-human primate model of aGVHD, spatiotemporal profiling of gut-infiltrating pathogenic donor T cells demonstrated TRM-phenotype acquisition as early as 8 days post-transplant²²¹. However, these pathogenic TRM are transcriptionally distinct from homeostatic TRM and display strong upregulation of cytokine pathways, metabolic reprogramming and an activated profile, which is induced by combined upstream signaling of IL-15 and IL-21²²¹.

Clone tracking of CD4+ and CD8+ T cells in mouse models of GVHD uncovered uneven distribution of alloreactive T cell clones across target tissues early during disease development¹⁵⁶. With the progression of disease, the clone repertoire in different tissues becomes more dissimilar, and pathogenic T cell clones are maintained within affected tissues without replenishment of circulatory T cells. A small subset of TCF1+ progenitor like T cells maintains the alloreactive TRM population within target tissues¹⁵⁶. Moreover, effector CD8+ T cells are reprogrammed by tissue-specific cues after recruitment, overwriting their lymph node-instructed profiles¹⁹³. These findings indicate that pathogenic mechanisms in aGVHD occur locally rather than systemically with little input from circulating T cells after initial disease development.

A recent study in humans corroborates this hypothesis. DeWolf et al sampled multiple autopsy tissues from patients with steroid-refractory GVHD and performed TCR sequencing, confirming that, even after prolonged GVHD, TCR repertoires are rather tissue-specific with the greatest overlap in anatomically related tissues, such as small and large intestine²²². These

studies underscore the importance of investigating pathologic immune processes within the tissue and finding targeted therapies that can mitigate them in a tissue-specific manner.

Host TRM chimerism following HSCT and in GVHD

While the studies summarized in the previous paragraph focus on donor cells developing into TRM, the contribution of surviving host cells is often neglected in aGVHD development. Already in the late 2000s, it was demonstrated that certain myeloid cells are protected from radio-chemotherapy in the tissue, leading to long-term mixed host/donor chimerism of these cell types in some patients²²³. Importantly, the persistence of host myeloid APC is correlated with the development of aGVHD^{223,224}. However, for TRM this phenomenon was only recently investigated. Our lab was among the first to show that, while circulating immune cells are virtually completely donor-derived after transplantation, roughly 50% of host TRM survive myeloablative conditioning¹³⁹. After donor cell engraftment these host TRM are not replaced by donor cells but maintained in the skin even 10 years after treatment. Interestingly, individuals later developing aGVHD have elevated levels of activated Ki67⁺ TRM in the skin even before receiving the donor graft¹³⁹. Another study reported the existence of activated host T cells in skin and gut aGVHD lesions in humans¹⁴⁰.

The contribution of host TRM to aGVHD development is a completely novel concept and warrants further investigation to understand the exact mechanisms of host TRM-mediated pathogenic signaling. Dissecting these mechanisms will improve our understanding of disease development and might provide new approaches for targeted prevention and therapy in aGVHD.

1.4. Aims of this thesis

Years of immunological research on mice and humans have highlighted differences between the two species, especially in organ physiology and immunology^{225,226}, underlining the importance to investigate disease pathologies, such as aGVHD, in human samples. T cells from peripheral blood have been extensively studied in the context of aGVHD. However, the notion of host TRM contributing to aGVHD pathology is an exciting research perspective, which could lead to the discovery of new biomarkers or therapeutic interventions for aGVHD development. Furthermore, the observation that host TRM are spared by myeloablative conditioning highlights the potential to study mechanisms of TRM longevity and persistence in human tissue samples in this special patient cohort. Per definition, host TRM were generated before transplantation, hence, long-term follow-up studies allow tracing of host TRM for years after transplantation and examination of their phenotype, function and migration patterns.

Harnessing the power of large datasets to create models of disease development and progression is becoming increasingly relevant in clinical research. High-dimensional single-cell technologies have emerged as powerful tools to detect rare sub-populations of cells with unique functions that might drive (disease-)specific phenotypes. In this thesis, we aim to use these tools to investigate TRM biology following HSCT and during aGVHD. With the ability to distinguish host and donor origin of immune cells we expect to find disease-, as well as genotype-specific cell states, of which we can extract differential gene expression profiles. Integration of transcriptional information, T cell clonality, tissue source and host/donor origin, gives us the unique opportunity to create a high-resolution map of T cell fate early after HSCT and in aGVHD. To better understand human TRM biology, we formulated the following aims for this thesis:

Aim 1 – Dissecting cellular dynamics of donor TRM formation in the skin

Aim 2 – Characterizing the phenotypic and functional heterogeneity of host and donor TRM

Aim 3 – Investigating retrograde migration potential of host and donor TRM following HSCT

2. Results

2.1. Prologue

TRM were initially thought to be terminally differentiated cells, that, once residency is established, permanently remain in the skin without any recirculation to the lymph or blood stream. However, in recent years this concept was challenged, as several groups could show in murine adoptive transfer and parabiosis models, that TRM of the skin and other organs have recirculation potential^{89,90,98}. A subsequent study in humanized mice and healthy human subjects indicated that this phenomenon is not unique to mice⁹¹. As described in the introduction, longitudinal tissue sampling of HSCT patients offers a unique opportunity to study TRM biology in the human system. As we previously observed an association of host TRM with the development of aGVHD in these patients, we set out to I) define the recirculation potential of bona fide host TRM in HSCT patients after transplantation and to II) investigate whether this mechanism of TRM recirculation could be implicated in aGVHD pathogenesis even in organs other than the skin. In the following study we showed that so called cTRM (circulating T cells with skin tissue-resident memory phenotype) can be detected in the blood of patients after HSCT, that they are enriched in patients with active cutaneous aGVHD and that they display pro-inflammatory properties and may seed aGVHD target organs other than the skin, suggesting a contribution to the propagation of systemic aGVHD inflammation. The graphical abstract published with this study is shown in Figure 4.

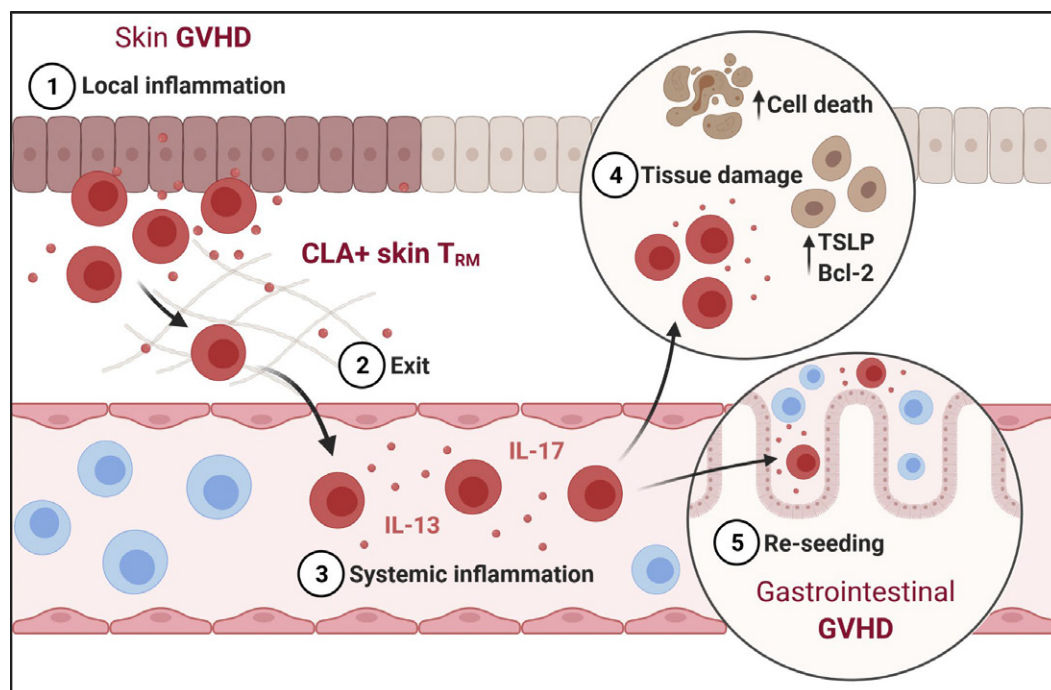


Figure 4 – Graphical abstract published with the article “Human resident memory T cells exit the skin and mediate systemic Th2-driven inflammation”

2.1.1. Human resident memory T cells exit the skin and mediate systemic Th2 driven inflammation



ARTICLE

Human resident memory T cells exit the skin and mediate systemic Th2-driven inflammation

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Emigration of tissue-resident memory T cells (TRMs) was recently introduced in mouse models and may drive systemic inflammation. Skin TRMs of patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) can coexist beside donor T cells, offering a unique human model system to study T cell migration. By genotyping, mathematical modeling, single-cell transcriptomics, and functional analysis of patient blood and skin T cells, we detected a small consistent population of circulating skin-derived T cells with a TRM phenotype (cTRMs) in the blood and unveil their skin origin and striking resemblance to skin TRMs. Blood from patients with active graft-versus-host disease (GVHD) contains elevated numbers of host cTRMs producing pro-inflammatory Th2/Th17 cytokines and mediating keratinocyte damage. Expression of gut-homing receptors and the occurrence of cTRMs in gastrointestinal GVHD lesions emphasize their potential to reseed and propagate inflammation in distant organs. Collectively, we describe a distinct circulating T cell population mirroring skin inflammation, which could serve as a biomarker or therapeutic target in GVHD.

Introduction

Tissue-resident memory T cells (TRMs) play a vital role for the local immune milieu, facilitating immune homeostasis within the tissue and rapid recall responses in recurring infections (Mueller and Mackay, 2016). TRMs are largely independent from the circulatory T cell pool and are identified by the expression of residency markers, including the S1P1-suppressor CD69 and the Integrin α E (CD103; Clark et al., 2006; Kumar et al., 2017; Watanabe et al., 2015).

Originally, TRMs were regarded as a terminally differentiated subset of memory T cells that, once generated in the tissue, were permanently located there, with limited motility and local proliferation (Beura et al., 2018; Park et al., 2018). However, this concept was challenged in murine experimental models that described a retrograde migration of cells with a TRM phenotype to draining lymph nodes, the blood, and secondary tissue sites (Behr et al., 2020; Beura et al., 2018; Fonseca et al., 2020; Klicznik et al., 2019). Klicznik et al. (2019) employed a humanized skin transplant mouse model to demonstrate that human skin-derived CD4⁺ T cells with a TRM signature, identified by expression of the cutaneous lymphocyte-associated antigen

(CLA) and CD103, can down-regulate CD69, exit the skin into the circulation, and reseed a distant skin site. Fonseca et al. (2020) recently revealed an unexpected developmental plasticity of TRMs in a murine transplant model. Upon recall antigen challenge, CD8⁺ TRMs reentered the circulation and trans-differentiated into central memory-like T cells while maintaining the propensity to home back to their tissue of origin and regain a TRM phenotype.

Despite recent reports of T cells with a skin TRM signature in human lymph and blood (Klicznik et al., 2019), definitive proof of (re)circulating TRMs as a separate entity in the human system remains elusive. As TRMs have been shown to play a pathogenic role in numerous inflammatory diseases (Chen and Shen, 2020), potential skin exit, trans-differentiation, and entry into distant tissues by disease-driving TRMs may represent a novel systemic factor contributing to these diseases. It is therefore eminent that the presence and role of circulating TRM-like cells warrant further investigation in the human setting.

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Recently, we and others demonstrated in patients receiving allogeneic hematopoietic stem cell transplant (HSCT) that host-derived skin TRMs are maintained in the skin for at least 10 yr, whereas the circulating T cell pool is rapidly replaced by the donor T cells (Divito et al., 2020; Strobl et al., 2020b). Importantly, activated host T cells were detected in lesional skin and in the gut of graft-versus-host disease (GVHD) patients, suggesting that they play a role in GVHD and drive an inflammatory host-versus-graft reaction. Based on those findings, the unique clinical setting of allogeneic HSCT provides an ideal model to study (re)entry of human skin TRMs into the circulation.

Results

Circulating CD4⁺ T cells with a skin-resident phenotype are found in the blood of immune cell-reconstituted patients after HSCT

In the course of HSCT, peripheral blood T cells are largely eliminated by conditioning treatments and return to baseline levels weeks to months after transplantation of donor peripheral blood stem cells (PBSCs; Strobl et al., 2020b; Fig. 1 A). To investigate marker genes for circulating T cells with a skin TRM phenotype (cTRMs; Klicznik et al., 2019) in HSCT, we FACS-sorted T cells of 11 patients undergoing HSCT at five time points: B, baseline; Tx, hours before transplantation after completion of conditioning treatment; 14, day 14 after Tx; 100, day 100 after Tx; and 365, 1 yr after Tx; (Fig. S1, Cohort 1). RNA sequencing (RNA-seq) of 100 bulk T cells per time point revealed stable expression of *SELPLG*, the gene coding for PSLG-1, which can be post-translationally modified to harbor the E-selectin-binding epitope of CLA on skin-homing T cells (Fuhlbrigge et al., 1997) and continuous expression of *ITGAE* (CD103) at a low level (Fig. 1 B). In contrast, *ITGA1* (CD49a), a marker for cytotoxic TRMs (Cheuk et al., 2017), was not consistently detectable by RNA-seq (Fig. S2 A). To evaluate CLA and CD103 on protein level, we checked for surface expression of these cTRM markers in T cells in the blood after immune reconstitution. In accordance with RNA expression, we detected a small population of CLA⁺ and CD103-expressing cells among CD4⁺ and CD8⁺ T cells (Fig. 1, C and D; and Fig. S1, Cohort 2). The vast majority of CD4⁺ T cells were CD103 negative and were termed conventional T cells (T_{CONV} cells), and a minor fraction of CD4⁺ CD103⁺ T cells were CLA⁺ (Fig. 1, C, D, and F). In contrast, the proportions of CLA⁺CD103⁺ and CLA⁺CD103⁺ CD8⁺ T cells were equal, indicating a preferential egress of CD4⁺ TRMs from the skin in these patients (Fig. 1 F). Skin TRMs commonly express S1PR1-antagonist CD69, which is used as an activation marker of peripheral blood T cells (Cibrián and Sánchez-Madrid, 2017; Mackay et al., 2015). We found only a minor fraction of CD4⁺ or CD8⁺ cTRMs expressing CD69 (Fig. 1 I), which is consistent with Klicznik et al. (2019) reporting that CD69 down-regulation is required for skin egress. CD4⁺ cTRMs were also largely CD49a⁺, whereas a higher percentage of CD8⁺ cTRMs expressed CD49a, in line with their cytotoxic function (Cheuk et al., 2017; Fig. 1 I). Pearson correlation analysis found no association between percentage of cTRMs and time after Tx in CD4⁺ or CD8⁺ T cells (Fig. 1 E), arguing for a stable subset of

cTRMs over time. To confirm that cTRMs represent memory $\alpha\beta$ T cells, we stained for CD45RO as a memory marker and TCR- $\alpha\beta$ (Fig. 1, G and H). The vast majority of cTRMs from both CD4⁺ and CD8⁺ T cells were positive for both markers (Fig. 1 H). Since CD4⁺ cTRMs seem to be enriched in our patients compared with reported values in healthy controls (<0.2% of CD4⁺ T cells; Klicznik et al., 2019) and compared with CD8⁺ cTRMs, we focused further experiments on CD4⁺ cTRMs and refer to them as cTRMs throughout the manuscript.

cTRMs are host derived and detectable in skin before transplantation

In solid organ transplantation, resident T cells of gut, kidney, or skin may remain stationary in their respective tissue and are thus cotransplanted with the donor organ into the recipient's body (Bartolomé-Casado et al., 2019; de Leur et al., 2019; Lian et al., 2014). Inversely, in the unique setting of allogeneic HSCT, TRMs survive conditioning treatment due to altered metabolism and senescent features and coexist with donor T cells, forming a human chimeric host (Divito et al., 2020; Strobl et al., 2020b). To our knowledge, the similarity of human cTRMs to their counterparts in tissues has exclusively been identified by phenotypic features, but proof of the relation between the two compartments in humans is missing. We therefore tracked XX/XY genotypes of T cells after sex-mismatched transplantation to distinguish host and donor cells (Fig. 2 A; and Fig. S1, Cohort 2). As expected after successful HSCT, the overwhelming majority (mean, 88.01%) of T_{CONV} cells were of donor origin (Fig. 2 B). Patients with higher levels of host-derived T_{CONV} (Fig. 2 B, triangle symbols) suffered from skin or mucosal infections at the time of blood sampling (skin infection with HSV or varicella zoster virus and viral sinus infection), providing insights into mobilization mechanisms of TRMs into peripheral blood. Strikingly, analysis of sorted cTRMs revealed that a mean of 50% of cTRMs exhibited a host genotype (Fig. 2 B).

To address the chimerism in other memory populations in the peripheral blood, we sorted T cells according to their naive (T_{NAIVE} = CD45RA⁺ CD62L⁺), central memory (T_{CM} = CD45RO⁺ CD62L⁺), effector memory (T_{EM} = CD45RO⁺ CD62L⁺), and terminally differentiated effector memory (T_{EMRA} = CD45RA⁺ CD62L⁺) phenotypes (Fig. S2) and analyzed the XX/XY genotype. As expected, the vast majority of T_{NAIVE} cells were of donor origin (Fig. 2 C). Importantly, the proportion of host-derived cells among memory T cell subsets (T_{CM} and T_{EMRA} cells) was minor (Fig. 2 C). In contrast to overall cTRMs (Fig. 1 E), host-derived cTRMs showed a trend to slowly decline in two phases, with a faster decay within the first 100 d (Fig. 2 D).

To investigate emergence of TCR clones in contrast to persistence of TRM and cTRM clones, we performed TCR sequencing from matched pre- and post-transplantation skin and blood samples (Fig. 2 E). In both skin and blood, a mean of 1% of clones were shared between pre- and post-transplantation. Interestingly, 0.4% of clones present in blood after transplantation were found in pretransplant skin, thus cross-shared between tissues and time points (Fig. 2 F and Table S4). This fraction of cross-shared host-derived clones insignificantly declined over time (Fig. S2 C).

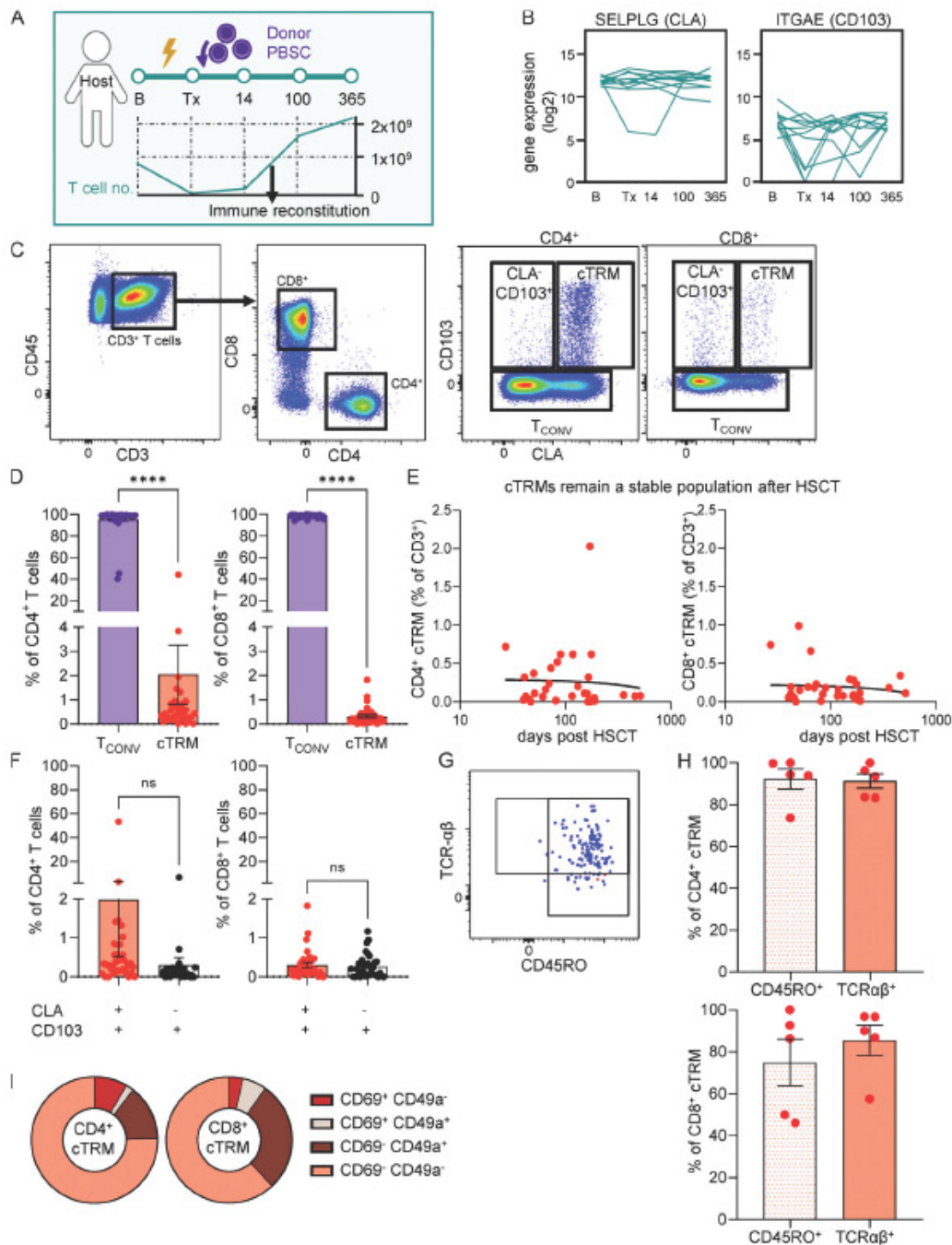


Figure 1. Circulating CD4⁺ T cells with a skin-resident phenotype are enriched in the blood of immune cell-reconstituted patients after HSCT. (A) Graphical representation of sampling timeline and blood T cell no./liter before initiation of conditioning (B); hours before transplantation after completion of conditioning treatment (Tx); and 14, 100, and 365 d after Tx (14, 100, and 365; n = 11). (B) RNA-based progression scores of *SELPLG* and *ITGAE* in FACS-sorted bulk T cells. Data shown as mean expression in 100 T cells per patient per time point (n = 11). (C) Representative gating strategy and sorting gates for cTRMs and T_{CONV}. Cells were pregated as live single leukocytes. (D) Mean percentage (±SEM) of cTRMs and T_{CONV} among CD4⁺ and CD8⁺ T cells (n = 36). Statistical analysis was performed with paired Student's t test (****, P < 0.0001). (E) Linear regression of CD4⁺ and CD8⁺ cTRMs (percentage of all T cells) to time after Tx.

(days; $n = 31$; $CD4^+ R^2 = 0.0003755$, $CD8^+ R^2 = 0.01269$). Data plotted as percentage cTRM of total T cells versus days after Tx. One dot indicates one patient. (F) Mean percentage ($\pm$ SEM) of $CLA^+ CD103^+$ and $CLA^+ CD103^+$ cells among $CD4^+$ and $CD8^+$ T cells ($n = 36$). Statistical analysis was performed with paired Student's *t* test. (G and H) Representative gating plot (G) and mean percentage ($\pm$ SEM) of CD45RO and TCR- $\alpha\beta$ expression of $CD4^+$ and $CD8^+$ cTRMs ($n = 5$; H). (I) CD69 and CD49a surface molecule expression on cTRMs assessed by flow cytometry of PBMCs. Data shown as mean percentage of $CD103^+CLA^+$ T cells positive for the respective markers ($n = 21$).

Mathematical modeling confirms concept of cTRMs relating to skin TRMs

The observation that cutaneous host TRMs form a population of cTRMs raises the question of how the cTRM population is maintained after transplantation. Trans-differentiation from host T_{CONV} cells is rather unlikely due to the low numbers of host T_{CONV} in the blood at Tx (Fig. 2 B). Similarly, the novel donor cTRM pool cannot be formed from preexisting donor skin TRMs as donor T cells are only transferred on Tx and need to form a skin TRM population first. This leaves three possible routes of origin for the renewal of the host cTRM population (Fig. 3 D). Either the cTRM pool is maintained from the cutaneous TRM reservoir or it stems from self-renewal of cTRMs or trans-differentiation from T_{CONV} within the peripheral blood.

To assess the contribution of skin versus blood T cells to the cTRM pool after transplantation, we measured the number of skin and blood T cells surviving radio-chemotherapy (Fig. 3, A and B; and Fig. S1, Cohort 1). While $>10^{10}$ TRMs were residing in the skin of a patient at transplantation, $<4 \times 10^8$ T cells were present in peripheral blood (Fig. 3 C). Consistently, donor-derived peripheral blood mononuclear cells (PBMCs), including cTRMs, were efficiently depleted by conditioning therapy (Fig. S3 A, Cohort 4). We hypothesized that due to the high number of host TRMs in the skin compartment, host cTRMs are maintained by continuous low-level emigration of skin TRMs. To analyze this in detail and determine host and donor T cell cycles needed to maintain the cTRM pool, we created a symmetrical model system based on the measured saturation densities of cTRMs and T_{CONV} cells of host and donor origin (Fig. 3 D). We first established nonlinear differential equations to describe the change in T cell populations. The equations consider exponential growth for small populations and saturation for large populations. The host cTRM cycle comprises the change of the host skin TRM population (N_S) and the host cTRM population in blood (N_B), as well as the small circulating host T_{CONV} population (N_{CONV}). It depends on (i) the diffusion of skin TRMs to blood cTRMs (and vice versa) with a rate of Γ_{BS} ; (ii) the growth of respective skin and blood populations with rates Γ_S and Γ_B , the self-renewal rate of T_{CONV} (Γ_T); and (iii) trans-differentiation from conventional T cells, which acquire a cTRM phenotype (Γ_{TS}). This results in three equation terms:

$$\begin{aligned} \frac{\partial N_S}{\partial t} &= -\Gamma_{BS}[(N_B^{sat} - N_B - n_B)N_S - (N_S^{sat} - N_S - n_S)N_B] \\ &+ \Gamma_S(N_S^{sat} - N_S - n_S)N_S + \Gamma_{TS}(N_S^{sat} - N_S - n_S)N_{CONV}, \\ \frac{\partial N_B}{\partial t} &= +\Gamma_{BS}[(N_B^{sat} - N_B - n_B)N_S - (N_S^{sat} - N_S - n_S)N_B] \\ &+ \Gamma_B(N_B^{sat} - N_B - n_B)N_B, \\ \frac{\partial N_{CONV}}{\partial t} &= -\Gamma_{TS}(N_S^{sat} - N_S - n_S)N_{CONV} \\ &+ \Gamma_{CONV}(N_{CONV}^{sat} - N_{CONV} - n_{CONV})N_{CONV} \\ &+ \Gamma_P(N_{CONV}^{sat} - N_{CONV} - n_{CONV})N_{PBSC}. \end{aligned}$$

$$\begin{aligned} \frac{\partial N_{CONV}}{\partial t} &= -\Gamma_{TS}(N_S^{sat} - N_S - n_S)N_{CONV} \\ &+ \Gamma_{CONV}(N_{CONV}^{sat} - N_{CONV} - n_{CONV})N_{CONV}. \end{aligned}$$

As opposed to the host cycle, the donor T cell population also grows by differentiation from PBSCs to T_{CONV} with rate Γ_P . Therefore, we expect changes in donor T cell populations of transplanted PBSCs (n_{PBSC}), conventional T cells (n_{CONV}), skin TRMs (n_S), and cTRMs (n_B) by differentiation, egress from/to tissue, and proliferation rate.

$$\begin{aligned} \frac{\partial n_S}{\partial t} &= -\Gamma_{BS}[(N_B^{sat} - N_B - n_B)n_S - (N_S^{sat} - N_S - n_S)n_B] \\ &+ \Gamma_S(N_S^{sat} - N_S - n_S)n_S + \Gamma_{TS}(N_S^{sat} - N_S - n_S)n_{CONV}, \\ \frac{\partial n_B}{\partial t} &= +\Gamma_{BS}[(N_B^{sat} - N_B - n_B)n_S - (N_S^{sat} - N_S - n_S)n_B] \\ &+ \Gamma_B(N_B^{sat} - N_B - n_B)n_B, \\ \frac{\partial n_{CONV}}{\partial t} &= -\Gamma_{TS}(N_S^{sat} - N_S - n_S)n_{CONV} \\ &+ \Gamma_{CONV}(N_{CONV}^{sat} - N_{CONV} - n_{CONV})n_{CONV} \\ &+ \Gamma_P(N_{CONV}^{sat} - N_{CONV} - n_{CONV})n_{PBSC}. \end{aligned}$$

The two cycles are coupled by requiring that the growth saturates when the total number of T_{CONV} cells, TRMs, and cTRMs equals that of a healthy individual given by N_{CONV}^{sat} , N_S^{sat} , and N_B^{sat} , respectively. By solving the differential equations for various rates and by analytical approximations, we find that the experimental observation of an equal number of host and donor cTRMs (Fig. 2 C) is met when the diffusion from host skin TRMs to host cTRMs is faster than the proliferation of host cTRMs in blood.

We next tested our equation system by adopting potential cell proliferation and migration rates and plotting them using Mathlib package for Jupyter Notebook. When plotting relative cell proliferation rates based on the proliferation of conventional T cells ($\Gamma_T = 1$), where $\Gamma_B = \Gamma_T$, $\Gamma_P = 10$, $\Gamma_{BS} = 0.5$, $\Gamma_{TS} = 0.5$, and $\Gamma_S = 0$, we saw achievement of the experimentally observed relative cell numbers within the first weeks after transplantation (Fig. 3 E, Scenario 1). Other scenarios presuming higher cTRM proliferation rates ($\Gamma_B \geq 2$) or rapid adoption of a cTRM phenotype by T_{CONV} ($\Gamma_{TS} \geq 2$) result in unrealistic growth curves (Fig. S3 B, Scenarios 3 and 4). Tested cell cycling rates are shown in Fig. S3 C. Interestingly, due to high skin cell numbers, proliferation of cutaneous TRMs ($\Gamma_B > 0$) would result in rapid saturation of the cTRM pool with host-derived T cells (Fig. 3 E, Scenario 2), which is not the case according to our genotyping experiments (Fig. 2 B).

Thus, we can conclude that while the donor cTRM pool is formed from PBSCs via T_{CONV} and consequently developed

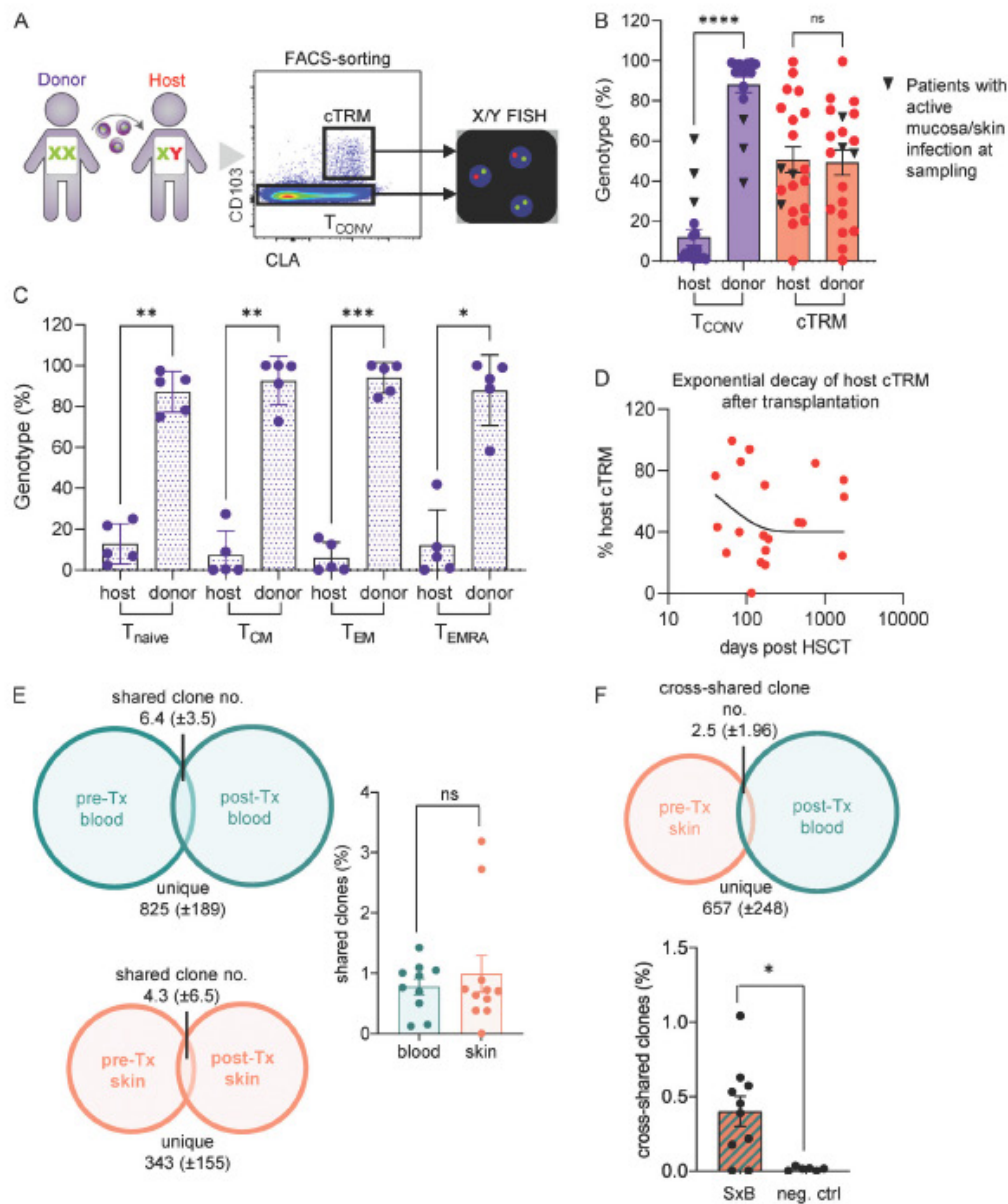


Figure 2. Host-derived cTRMs share clones detectable in the skin before transplantation. (A) Graphical representation of sample processing after sex-mismatched HSCT. (B and C) Host/donor genotype of T_{CONV} ($n = 18$) and cTRMs ($n = 20$; B) and T_{naive}, T_{CM}, T_{EM}, and T_{EMRA} ($n = 5$; C) as determined by XX/XY-FISH. Data shown as mean percentage ($\pm$ SEM) of detected T cells per sample after sorting of minimum 5,000 cells. One dot represents one patient; statistical analysis was performed with repeated measures one-way ANOVA with multiple comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (D) Correlation trend of host genotypes among cTRMs to the time after HSCT in a two-phase exponential decay model ($n = 20$; decay span 1: ~ 46.87 d, decay span 2: 49.10 d, ns). Following initial decay estimated at $K1 \sim 5.344e^{-0.15}$, half-life of host cTRMs was determined at 39 d ($K2 = 0.01776$). Data shown as percentage of host cTRMs among all cTRMs and days after Tx. (E) Sorted bulk T cells from 11 patients from pre- and post-transplant time points from skin and blood were RNA sequenced (Smart-seq2) and analyzed for their TCR repertoire by TRUST. Graphical representation of definition of blood and skin TCR clones that are shared between pre- and post-transplant time points and percentage of shared TCR clones of pre-Tx blood versus post-Tx blood ("blood") and pre-Tx skin versus post-Tx skin ("skin"). (F) Cross-shared clones from pre-Tx skin to post-Tx blood. Graphical representation and percentage of cross-shared clones from all clones ("SxB") and percentage of cross-shared clones in blood of different individuals at day 100 after transplantation ("neg. ctrl"). Data shown as mean percentage $\pm$ SEM of all detected TCR clones in each sample ($n = 11$). Statistical analysis in E and F was performed with paired (E) and unpaired (F) Student's t test (*, $P < 0.05$).

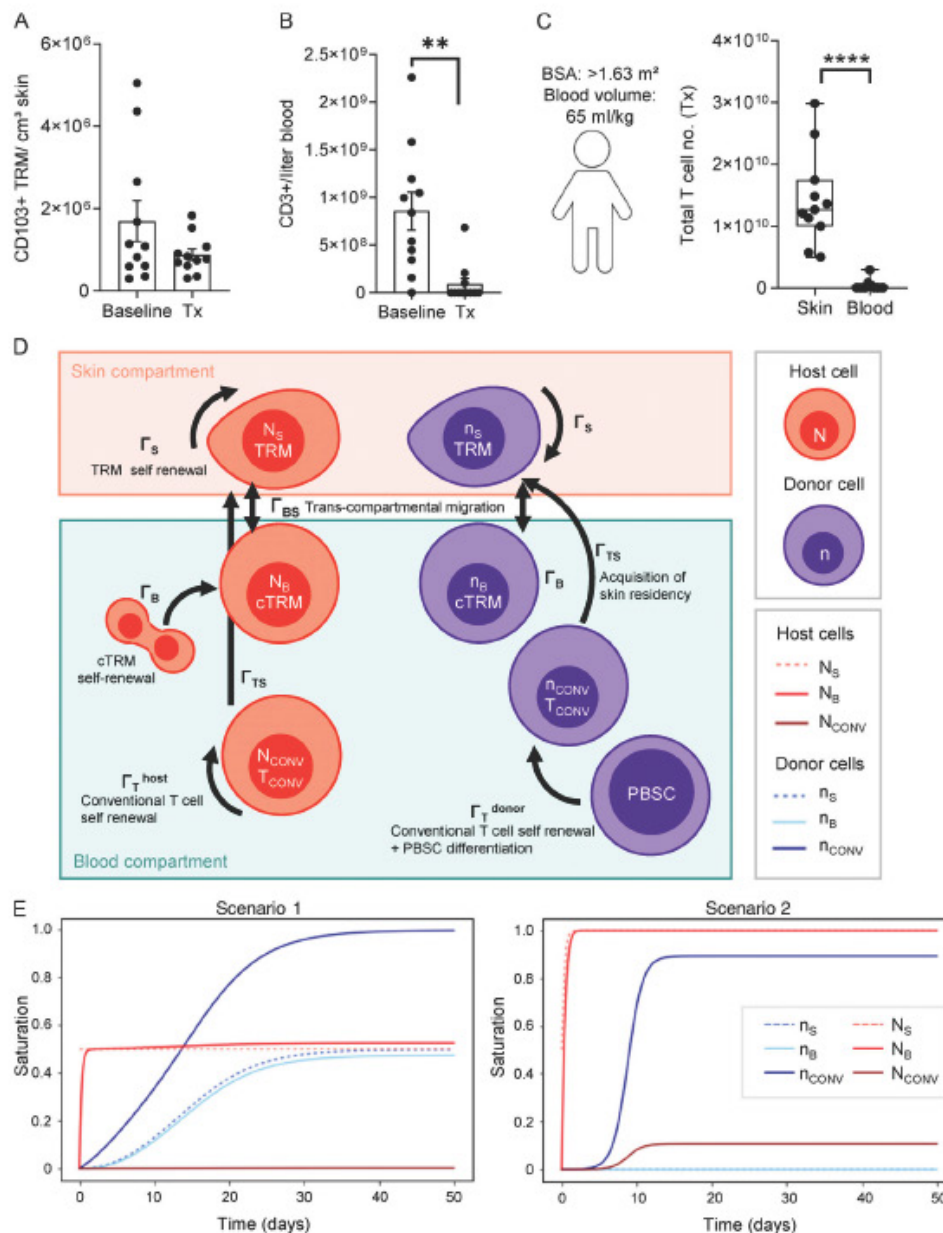


Figure 3. Mathematical modeling postulates relation of cTRMs to skin TRMs. (A) TRM number in skin before and after conditioning therapy as determined by immunofluorescence staining. Data shown as mean number ($\pm$ SEM) of CD103⁺CD3⁺ cells per cubic centimeter of skin at B and Tx ($n = 11$). (B) T cell number in peripheral blood before and after conditioning therapy as determined by flow cytometry. Data shown as mean number ($\pm$ SEM) of CD3⁺ lymphocytes per liter of blood at B and Tx, $n = 11$. (C) Total number of T cells in adult human skin and blood after conditioning before infusion of donor PBSCs. Data shown as mean number and range of cells at Tx calculated for an average human with a body surface area (BSA) of ± 1.63 m² and a blood volume of 65 ml/kg using the Du Bois formula ($BSA = 0.007184 \times W^{0.425} \times H^{0.725}$). Statistical analysis in B and C was performed with paired Student's *t* test; **, $P < 0.01$; ****, $P < 0.0001$. (D) Graphical representation of potential routes for maintenance of host/donor cTRMs and equation terms. (E) Modeling of symmetrical differential equation model by applying different cell proliferation and migration rates shown in Fig. S3 C. Scenario 1 (left panel) results in realistic cell saturation values; Scenario 2 (right panel) shows rapid saturation of the cTRM and TRM niches with host-derived cells. Saturation represents the sum of relative host and donor cells (1 = 100%). Time shown in days.

donor TRMs, the host cTRM pool is likely maintained by self-renewal from nonproliferating skin TRMs.

Single-cell RNA-seq unravels gene expression of host- and donor-derived circulating T cells after HSCT

To further characterize circulating host-derived T cells over time, we performed index-sorted single-cell flow cytometry and RNA-seq of T cells isolated from blood after HSCT (Fig. S1, Cohort 1). To increase the likelihood of isolating host-derived T cells, we sequenced peripheral blood T cells of one patient with slow donor T cell recovery and a mixed T cell chimerism at days 14 and 250 after HSCT (Fig. 4, A–D, patient 1; and Table S2) and one patient with normal donor T cell recovery at day 14 only, who had a full donor chimerism of T cells at later time points (Fig. S4, A, patient 2; and Table S3). At both time points, we saw two transcriptionally distinct populations of T cells (Fig. 4, A and C; and Fig. S4 A). Based on unsupervised t-distributed stochastic neighbor embedding (t-SNE) clustering of cells and confirmation by single-nucleotide polymorphisms (SNPs) identified in pretransplant samples, we were able to determine host and donor origin of T cells. SNP calling revealed that host cells were present in all samples and that cells clustered according to host/donor origin (Fig. 4, C and D). Notably, fewer SNPs of host cells were qualified (Fig. 4, C and D) due to low overall numbers of host-derived cells in combination with strict selection criteria (SNPs present in three or more host cells and absent in all donor-derived cells). SNP-based distinction of cells allowed differential gene expression analysis of donor versus host cell transcriptomes, enabling us to investigate differential expression of TRM marker genes from the two patients at day 14 (Fig. 4 E). Interestingly, we found host cells to have a gene expression profile relating to a cTRM phenotype, with a trend of higher expression of cTRM markers *ITGAE* (CD103), *SELPLG* (encoding for CLA), *LGALS3* (encoding for Galectin-3), and *PRDMI*, which have been associated with TRMs (de Almeida et al., 2020 Preprint; Mackay et al., 2016; Strobl et al., 2020b). Host cells also displayed significantly higher levels of *FABP5*, encoding for the fatty acid-binding protein 5 known to be crucial for skin TRM metabolism (Pan et al., 2017). *RUNX3*, a transcription factor associated with TRM (Milner et al., 2017) and cytotoxic function (Shan et al., 2017), and markers of circulating cells (*SIPRI* and *CCR7*; Mueller and Mackay, 2016) were expressed similarly between host and donor cells.

A population of proliferating cTRMs is found in blood of GVHD patients

Increasing evidence points toward a pivotal role of skin TRMs in GVHD pathogenesis (Divito et al., 2020; Strobl et al., 2020b). Studies in mice previously described that activation of skin TRMs during infection leads to skin exit and recirculation (Behr et al., 2020). Based on this, we stratified our patient cohort (Fig. S1, Cohort 2, sex-mismatched HSCT) into patients without GVHD or infection, with active GVHD, and with active viral skin infection at the time of sampling (Fig. 5 A). While percentages of host cTRMs in patients without GVHD development were maintained at ~25% of total cTRMs, patients with GVHD showed markedly higher percentages of host cTRMs of >60% (Fig. 5 B).

Interestingly, patients with viral skin infections such as varicella zoster virus or HSV had equally high percentages of host-derived cTRMs, which may indicate a recall response of long-term skin-resident T cells in the event of antigen encounter.

To better characterize the cTRM population in the blood, we sampled PBMCs of two patients with active GVHD (Fig. S1, Cohort 3) and performed high-throughput single-cell RNA-seq using the 10x Genomics protocol (Fig. 5 C). The overwhelming majority of cells were T cells, followed by subsets of myeloid cells, non-T lymphocytes (B cells, natural killer cells), and erythrocytes (Fig. 5 D). As cTRMs show a transcriptome distinct from T_{CONV} (Fig. 4) and express *CD103* on their surface (Fig. 1 C), we searched for T lymphocytes with high expression of *ITGAE* (coding for CD103). Indeed, a small population of T cells (Fig. 5 C, Cluster 13) fit this characteristic (Fig. 5 E). Importantly, these cells showed high expression for the proliferation marker *MKI67*. We also found increased expression of protein-coding genes for fatty acid-binding protein 5 (*FABP5*) and *LGALS3*, as well as the transcription factors *RUNX3* and *PRDMI* (Fig. 5 E), all previously described in TRMs (de Almeida et al., 2020 Preprint; Milner et al., 2017; Pan et al., 2017; Strobl et al., 2020b). The majority of cells in this cluster expressed genes encoding for the $\alpha\beta$ -TCR (Fig. S4 B), confirming our previous results (Fig. 1, G and H). The integrins *ITGA4* and *ITGB7* were both highly expressed in the cTRM cluster (Fig. 5 E). *ITGB7* codes for the $\beta 7$ integrin, which can either form a heterodimer with *CD103* to bind E-cadherin or with *ITGA4* to form the integrin $\alpha 4\beta 7$, which is important for gut homing. Expression of *ITGA4* within the cTRM cluster indicates the potential of the cells to home to other organs than the skin. Moreover, we detected expression of transcription factor *GATA3* in cTRMs, which regulates transcription in T helper 2 (Th2) cells. Thus, in GVHD, cTRMs form a proliferative population of Th2-type cells while maintaining a classical TRM transcriptome. These findings suggest that cTRMs are actively proliferating in the blood of patients with GVHD and could contribute to the disease phenotype.

cTRMs are functionally competent in GVHD inflammation

In acute GVHD, skin infiltrate is dominated by Th2 cells, while in both acute and chronic GVHD, tissue damage is described to be largely mediated by Th1 and Th17 donor T cells (Brüggen et al., 2014; Hill and Koyama, 2020). To investigate the contribution of cTRMs to systemic GVHD inflammation, we sampled the blood of patients with active GVHD (Fig. 6 A). In line with our findings on the RNA level (Fig. 5 E), we detected higher percentages of $GATA3^+$ cTRMs compared with T_{CONV} cells (Fig. 6, B and C). The $CD4/CD8$ ratio was high among cTRMs and <1 in T_{CONV} , indicating a relative increase of $CD4^+$ compared with $CD8^+$ T cells in cTRMs (Fig. 6 D). We next measured cytokine production by cTRMs in patients with active GVHD (Fig. 6, E and F; and Fig. S5 A). Both IL-13- and IL-17-producing T cells were increased among cTRMs, while we observed a trend toward lower IFN- γ production by cTRMs (Fig. 6 F). To confirm the GVHD-promoting phenotype of cTRMs, we sorted cTRMs and T_{CONV} cells from three patients with active acute GVHD (Fig. S1, Cohort 3) and performed bulk RNA-seq. Pathway enrichment analysis of differentially expressed genes (DEGs) of cTRMs versus T_{CONV}

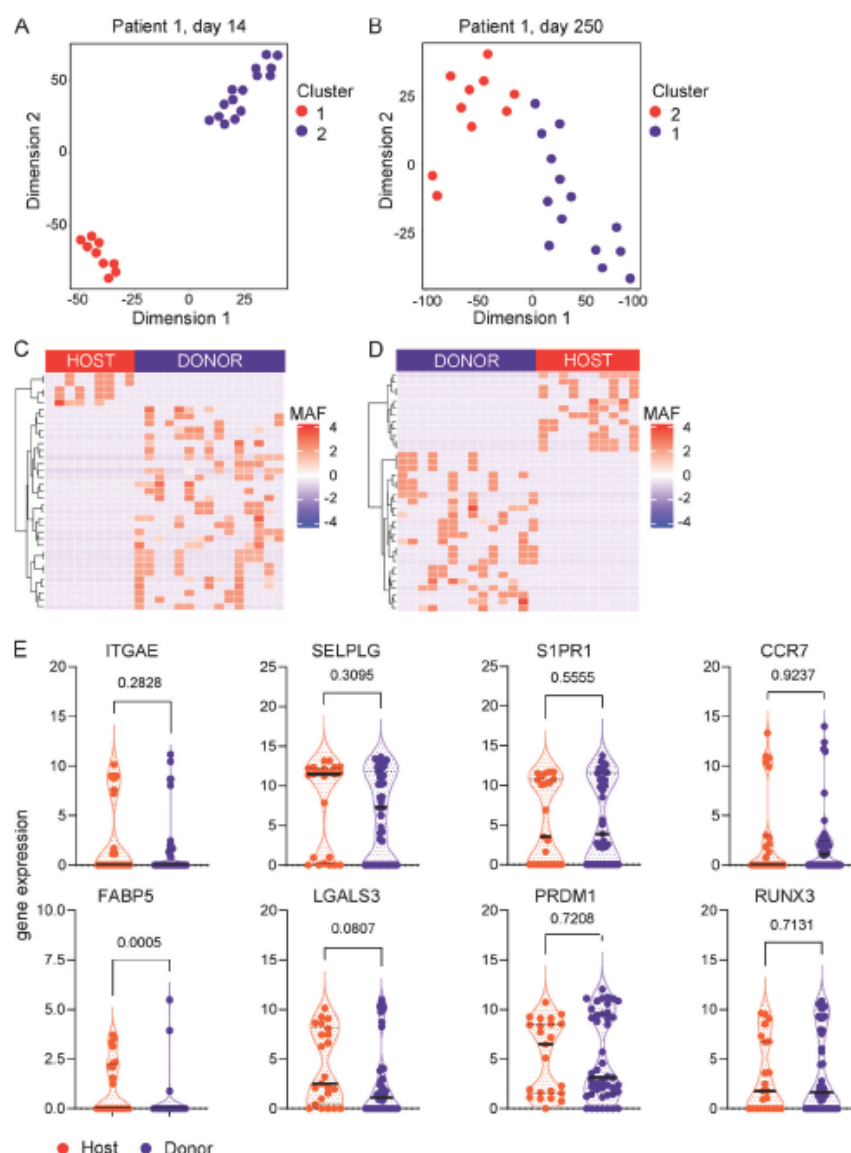


Figure 4. Single-cell RNA-seq unravels gene expression of host and donor-derived circulating T cells after HSCT. (A and B) Representative t-SNE plot of single blood T cells of patient 1 at day 14 (A) and day 250 (B) after HSCT as determined by RNA-seq according to the Smart-seq2 protocol. Unsupervised clustering resulted in two distinct clusters. One dot represents one T cell. (C and D) Allele frequency heat map of host- and donor-specific SNPs in cells of clusters 1 and 2 from A and B. (E) Expression of marker genes of interest by donor and host blood T cells at day 14 after HSCT (patients 1 and 2; see Fig. S4 A). Data shown as normalized expression ($\log_2$) value per cell. Solid bars indicate the median, and dashed lines show quartiles; one dot represents one cell. Statistical analysis was performed with Mann-Whitney test; P values are indicated in plots. MAF, mean allele frequency.

revealed that among the top 25 enriched pathways was GVHD itself, as well as several terms relating to GVHD and Th2/Th17-type inflammation, such as allograft rejection, asthma, inflammatory bowel disease, Th1 and Th2 cell differentiation, Th17 cell differentiation, or *Staphylococcus aureus* infection (Fig. 6 G). Moreover, we found up-regulation of genes linked to T cell activation (HLA Class II genes, *LCP2*), Th2 cell proliferation (*CD81*;

Maecker, 2003), and memory formation and effector function (*TLR2*; Komai-Koma et al., 2004; Fig. S5 B). Interestingly, *CD164*, a marker that is highly expressed on circulating and skin-tropic CD4⁺ T cells in patients with Sezary syndrome (Benoit et al., 2017), a form of cutaneous T cell lymphoma with a Th2 signature (Saed et al., 1994), was also up-regulated in GVHD cTRMs (Fig. S5 B). Genes previously associated with repression of Th17

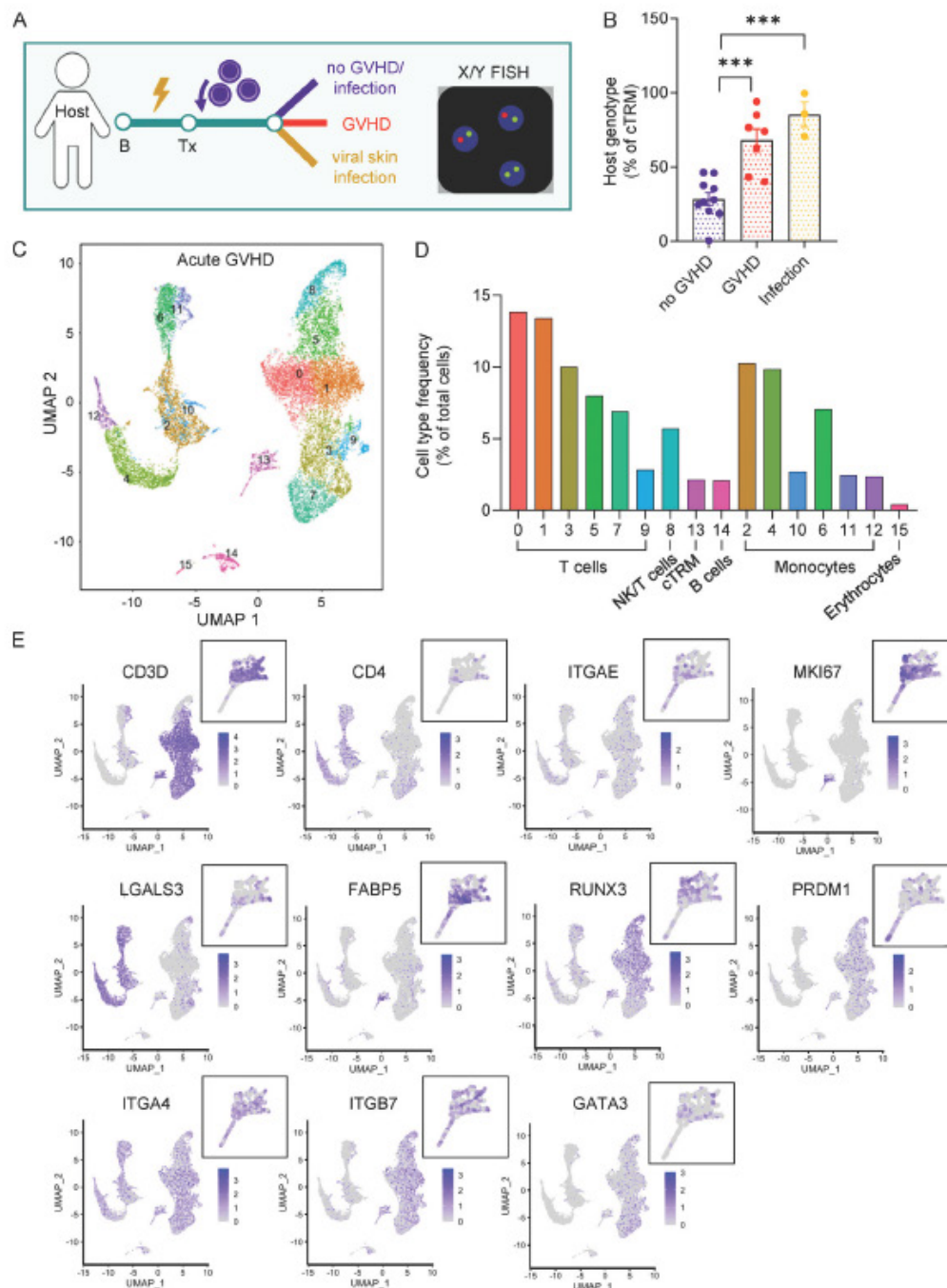


Figure 5. Characterization of cTRMs in GVHD patients. (A) Graphical representation of patient sampling and sample processing for B. (B) cTRM host genotype after HSCT in patients with/without GVHD and viral skin infections. Data shown as mean percentage ($\pm$ SEM) of host cTRMs of all cTRMs. One dot represents one patient, no GVHD ($n = 10$), GVHD ($n = 7$), infection ($n = 3$). Statistical analysis was performed by one-way ANOVA and Tukey's multiple comparisons (***, $P < 0.001$). (C) Combined UMAP plot of lymphoid and nonlymphoid PBMC populations in two patients with acute GVHD as determined by RNA-seq (10X Genomics). (D) Representation of immune cell populations in GVHD. Data shown as percentage of all cells. (E) Feature plots of marker expression of blood cells in GVHD. Data shown as log₂ expression for each cell. Zoom-in: cTRM population. NK, natural killer.

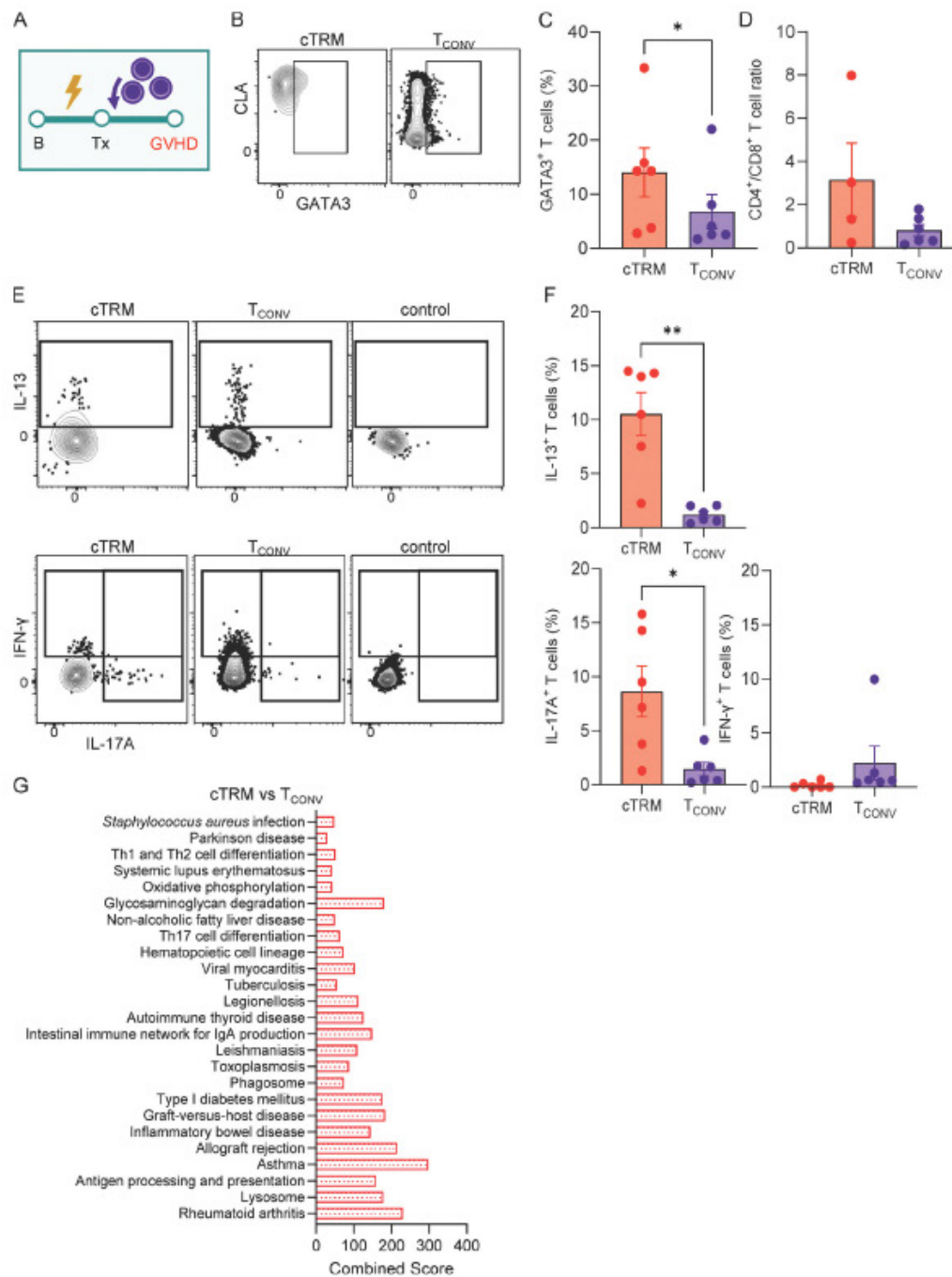


Figure 6. GATA3⁺ cTRMs are functionally competent in systemic GVHD inflammation. (A) Graphical representation of GVHD patient sampling (B and C) Representative gating plot (B) and mean percentage ($\pm$ SEM; C) of intracellular GATA3 expression of cTRMs versus T_{CONV} in GVHD patients (n = 6). (D) Mean ($\pm$ SEM) CD4/CD8 T cell ratio in cTRMs versus T_{CONV} in GVHD in blood samples determined by flow cytometry. (E and F) Representative gating plots (E) and mean expression ($\pm$ SEM) of IL-13, IL-17A, and IFN- γ cytokine expression in cTRMs versus T_{CONV} (F) in GVHD. Controls represent isotype or fluorescence-minus-one controls. Statistical analyses in C and F were performed by paired Student's t test (*, $P < 0.05$; **, $P < 0.01$; n = 6). (G) Top 25 enriched KEGG pathways in up-regulated genes ($P < 0.05$) in bulk RNA-seq of sorted cTRMs versus T_{CONV}. Bars indicate the combined enrichment score per pathway (n = 3).

responses (NOD2, ACKR2; Hansell et al., 2015; Napier et al., 2020) were down-regulated compared with T_{CONV} (Fig. S5 B). These findings indicate that systemic type 2 and type 17 responses in GVHD are at least partially driven by cTRMs.

cTRMs can exert damage and home to distant tissue sites during GVHD

To investigate if cTRMs are a byproduct of increased skin inflammation or can actively mediate damage in tissue GVHD, we sorted cTRMs and T_{CONV} cells from the blood of patients with GVHD (Fig. S1, Cohort 3), activated them with PMA/Ionomycin, and co-cultured them alone or in combination with keratinocytes isolated from healthy donor skin. We then checked for cell viability of keratinocytes, as well as expression of the pro-inflammatory cytokine TSLP and the anti-apoptotic molecule Bcl-2, since these markers were previously described to be elevated in keratinocytes in GVHD skin (Brüggen et al., 2014; Strobl et al., 2020a; Fig. 7 A). We observed that the presence of cTRMs leads to significantly more keratinocyte cell death compared with control, which was not the case for T_{CONV} cells (Fig. 7 B). Moreover, TSLP, as well as Bcl-2, was significantly up-regulated after co-cultures with cTRMs but not with T_{CONV} (Fig. 7 B). These data indicate that cTRMs can elicit tissue damage in the context of cutaneous GVHD.

Meanwhile, skin GVHD often precedes the involvement of other organs, including the gastrointestinal (GI) tract. Given that we found increased RNA expression of *ITGA4* and *ITGB7* on cTRMs in GVHD, we assessed in the blood of patients with active GVHD the presence of cTRMs expressing the gut-homing molecule $\alpha 4 \beta 7$ integrin (Fig. S1, Cohort 3). We found that a substantial portion (20–70%) of cTRMs coexpress $\alpha 4 \beta 7$ (Fig. 7, C and D). Among total $CD4^+$ T cells, $CLA^+CD103^+ \alpha 4 \beta 7^+$ cTRMs comprised <1% (Fig. 7 E). $CD8^+$ T cells displayed a similar pattern of $\alpha 4 \beta 7$ expression (Fig. S5, C and D).

Based on this, we examined gut biopsies of patients with active GI GVHD (GI-GVHD) for the presence of skin marker molecule-expressing TRMs (Fig. S1, Cohort 3). Indeed, we found that in all samples investigated a fraction of GI mucosa T cells expressed CLA in addition to CD103 (Fig. 7, F and G). Among the T cells coexpressing CLA and CD103, >99% were $CD4^+$ (Fig. 7 H). The presence of T cells with a phenotype consistent with skin-derived cTRMs in GI-GVHD emphasizes the role of cTRMs in systemic GVHD and their involvement in tissue inflammation of different organs.

Collectively, we show that host-derived cTRMs can be tracked in the blood of patients after HSCT and during GVHD, display an activated Th2/Th17-like phenotype, and can elicit keratinocyte damage and home to distant organ sites, thereby contributing to systemic inflammation and propagating disease.

Discussion

Since their discovery, TRMs have been defined as a rather homogeneous and differentiated T cell subset terminally located in the tissue. Recent findings from mouse models and humans have challenged this concept and demonstrated a previously unknown plasticity of TRMs, including the potential to reenter the

circulation and reseed distant organ sites (Behr et al., 2020; Fonseca et al., 2020; Milner et al., 2020). This has been proposed to be a mechanism for spreading tissue-specific immunity from the primary site of antigen encounter over large areas and organs. However, it also raises the possibility that, in the case of detrimental effects mediated by TRMs, pathology can be distributed to distant tissue sites. Here, we show for the first time that skin-derived cTRMs can be tracked as a discrete population in human blood over prolonged periods of time. We previously reported that host skin TRMs survive conditioning during HSCT, as opposed to circulating T cells. In the present study, we provide evidence that surviving host TRMs exit the skin after transplantation and give rise to a stable pool of cTRMs, which (i) retains in part a skin-resident transcriptional signature and functional profile, (ii) elicits a GVHD-like phenotype on healthy keratinocytes upon activation, and (iii) homes to other GVHD target organs, highlighting their potential in systemic immune reactions. While cTRMs were present among $CD4^+$ and $CD8^+$ T cells, $CD4^+$ cTRMs were enriched compared with $CD8^+$ cTRMs in our patient cohorts. This is in line with the reports from healthy human individuals (Klicznik et al., 2019) and might represent one important difference with mouse models, where $CD8^+$ TRMs are the dominant recirculating TRM type (Behr et al., 2020; Fonseca et al., 2020).

In murine models, cell origin can easily be tracked by congenic strains for generating host/donor chimera. In our system, we use a similar approach for tracking host and donor cells on a genetic level. By creating a mathematical model of T cell cycles and testing cell proliferation scenarios with multiple variables, we demonstrate that a blood origin of cTRMs after HSCT is highly unlikely, as exceedingly high proliferation rates of remaining host cTRMs would be required to repopulate the cTRM reservoir, which is virtually absent at the time of transplantation. We furthermore detected that the equal distribution of host- and donor-derived cTRMs is maintained only by relatively high proliferation rates of donor PBSCs and T_{CONV} cells and low or absent proliferation of host skin TRMs. This concurs with previous studies showing host-derived skin TRMs after HSCT to express markers for cellular senescence (Strobl et al., 2020b).

Naturally, our findings raise the question of how the emigration of TRMs from skin is orchestrated. According to the concept of recently described “outside-in” recall responses (Fonseca et al., 2020), in a HSCT scenario, TRMs may be activated during the tissue-damaging conditioning regimen and subsequently undergo retrograde migration to the circulation. In line with this hypothesis, we found an increased percentage of cTRMs in the blood of patients with GVHD and cutaneous infection. Previous studies proposed outside-in recall responses as a mechanism to promote host-protective immunity, but our results suggest that this mechanism could also lead to a systemic contribution to inflammatory diseases originally manifesting in tissues. The clinical relevance of circulating host-derived T cells in the pathogenesis of GVHD is supported by the association of mixed T cell chimerism with GVHD development (Mousavi et al., 2017). We found that cTRMs display a Th2/Th17 signature with increased expression of GATA3 and IL-13, as well as higher levels of IL-17 compared with T_{CONV} after stimulation. We

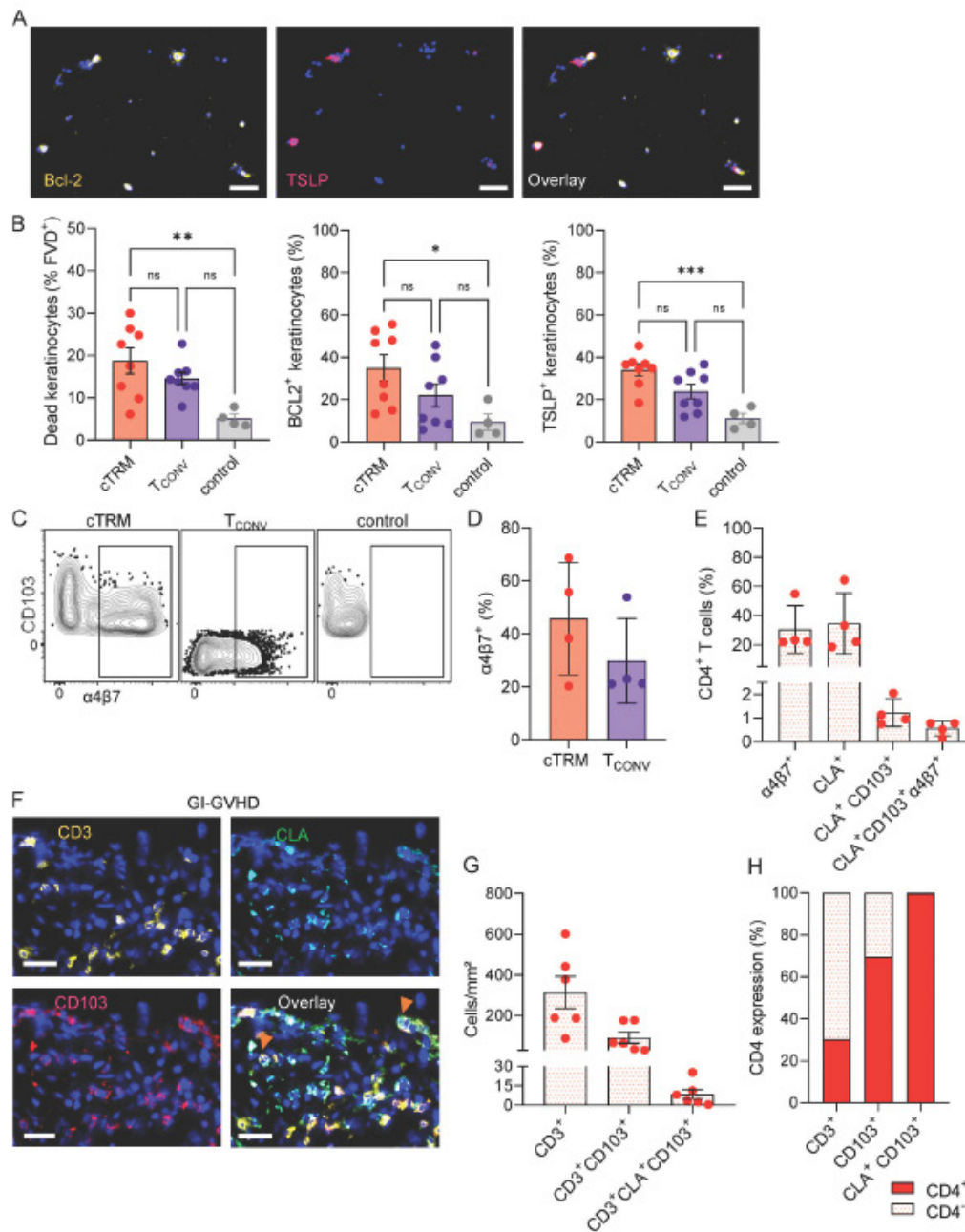


Figure 7. cTRMs can promote tissue damage and home to distant organ sites. (A) Representative immunofluorescence images of keratinocytes after co-culture with cTRM, stained with DAPI (blue), Bcl-2-APC (left), TSLP-PE (middle), and overlay (right); scale bar = 100 μ m. (B) Mean percentage ($\pm$ SEM) of dead keratinocytes (determined by LIVE/DEAD staining), Bcl-2, and TSLP expression of keratinocytes cultured alone (control) with cTRMs or with T_{CONV} at a ratio of 20:1, determined by immunofluorescence staining. Statistical analysis was performed by ordinary one-way ANOVA with multiple comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., $P > 0.05$; $n = 4$). (C and D) Representative gating plots (C) and mean percentage ($\pm$ SEM) of $\alpha 4\beta 7$ -integrin-expressing cTRMs and T_{CONV} from GVHD patients ($n = 4$; D). Control represents $\alpha 4\beta 7$ fluorescence-minus-one staining. (E) Mean percentage ($\pm$ SEM) of indicated markers among total peripheral blood-derived CD4⁺ T cells of GVHD patients ($n = 4$) determined by flow cytometry. (F) Representative immunofluorescence images of a GI-GVHD tissue sample stained with DAPI (blue), CD3-APC, CLA-FITC, and CD103-PE-Dazzle and overlay. Arrowheads indicate cTRMs; scale bar = 100 μ m. (G) Total number ($\pm$ SEM) of cells with labeled phenotype detected per square millimeter in gut biopsies of patients with acute GI-GVHD ($n = 6$). (H) Mean percentage of CD4⁺ and CD4⁻ cells in the indicated parent population in patients with acute GI-GVHD ($n = 6$), determined by immunofluorescence staining.

previously showed that Th2 cytokines are up-regulated in the skin during acute GVHD (Brüggen et al., 2014), and IL-17 has been reported to exacerbate cutaneous GVHD in mouse models (Mousavi et al., 2017). Of note, host-derived skin TRMs in GVHD lesions produce IFN- γ and IL-17 in situ, thereby promoting host-versus-graft immune responses (Divito et al., 2020). Interestingly, we did not observe increased IFN- γ production from cTRMs. In line with our findings, Divito et al. (2020) showed in a humanized mouse model of GVHD that IFN- γ was the only T cell-derived cytokine significantly decreased in the absence of donor T cells, suggesting that IFN- γ -mediated effects in GVHD can be largely attributed to donor T cells.

Keratinocyte damage mediated by cytokine-producing T cells in the skin is a hallmark of GVHD, and keratinocytes in acute GVHD display elevated levels of TSLP, a cytokine that promotes Th2 responses and can in turn be up-regulated by Th2 cytokines (Brüggen et al., 2014). Our finding of increased TSLP levels and enhanced cell death in healthy keratinocytes upon co-culture with activated cTRMs suggests that cTRMs can exacerbate tissue damage in GVHD and promote Th2 skewing in secondary tissue sites via TSLP signaling.

Alloreactive T cells expressing the gut-homing marker $\alpha 4 \beta 7$ integrin are known to promote GI-GVHD (Petrovic et al., 2004), and several studies indicate that treatment with the humanized $\alpha 4 \beta 7$ antagonist vedolizumab can ameliorate GI-GVHD (Coltoff et al., 2018). Here, we show that cTRMs in the blood of patients with GVHD express high levels of $\alpha 4 \beta 7$ and that cells with a cTRM phenotype can be detected in GI-GVHD. This is in line with murine CD8 $^{+}$ TRMs emigrating to the circulation and re-seeding different tissues such as the intestine, salivary gland, or female reproductive tract (Fonseca et al., 2020). Similar to this study, where ex-TRMs acquired a central memory phenotype before reexpressing TRM markers in the tissue, our data suggest that cTRMs partially change their TRM profile during recirculation but maintain crucial marker genes like FABP5. It remains to be determined where and by which signals cTRMs acquire gut-homing properties and how long a single cTRM remains in the circulation before tissue reentry.

In contrast to a CD8 $^{+}$ TRM recall-induced tissue exit, it was postulated that human CD4 $^{+}$ CLA $^{+}$ CD103 $^{+}$ T cells can exit the skin in steady state (Klicznik et al., 2019). Although cTRM numbers were increased during inflammation, we observed that the population in the blood remained stable over several years even in the absence of infection or cutaneous inflammation, indicating that human skin TRMs can undergo both proposed exit routes of recall-induced retrograde migration and steady-state emigration from skin. It will be important to determine in future studies what differences exist between homeostatic and activation-induced exit of cTRMs. Of note, it is likely that plasticity within the skin TRM population is not uniform, but that specific subsets are better equipped to exit the tissue.

Collectively, our study identifies human skin-derived CD4 $^{+}$ cTRMs as a stably maintained low-frequency T cell population in human blood with the potential to contribute to systemic inflammation and tissue damage at secondary target sites in the setting of GVHD. The present work highlights the power of single-cell technologies in addressing these questions, since we

could detect rare CD4 $^{+}$ blood cTRMs as a distinct population by their skin TRM-like transcriptome and surface molecules. Combining single-cell approaches for transcriptome, epigenome, and TCR sequencing will be a useful tool for elucidating the functional repertoire of cTRMs in the future.

Materials and methods

Patient sampling

For this study, we included patients presenting with myelodysplastic syndrome or acute or chronic leukemia receiving allogeneic peripheral blood HSCT. All patients participated in the study voluntarily and with fully informed written consent. The research is in line with national law and has been approved by the ethics committee of the Medical University of Vienna (ECS 1087/2016). Peripheral blood was sampled from four patient cohorts according to time to transplantation. For Cohort 1, patients were sampled at hospital admission before start of conditioning (B, $n = 11$), the day of HSCT (Tx, $n = 11$), at day 14 ($n = 14$), and after immune reconstitution between day 100 and day 365 after stem cell transfer ($n = 14$), and healthy-appearing skin (6-mm punch biopsy) was sampled at B ($n = 11$) and Tx ($n = 11$). For flow cytometric analysis and genotyping of cTRMs, patients were sampled after HSCT at the time of immune reconstitution (Cohort 2). From patients presenting with active skin GVHD, blood was sampled at disease onset before start of treatment with corticosteroids (Cohort 3). Additionally, intestinal biopsies of six patients with active gut GVHD were sampled at disease onset (Cohort 3). For cohort 4, we sampled five patients at B and the day of HSCT (Tx). GVHD was diagnosed according to the National Institutes of Health consensus criteria (Filipovich et al., 2005). Patient details are found in Table S5.

Flow cytometry and cell sorting

Single-cell suspensions of blood and skin were stained for surface or intracellular markers as previously described (Strobl et al., 2020a). For intracellular cytokine staining, PBMCs were stimulated for 4 h with IX Cell Activation Cocktail containing PMA/Ionomycin and Brefeldin A (Biolegend; 423303) in RPMI 1640 (Gibco; 52400025) according to the manufacturers' protocol. Antibodies used are indicated in Table S1. Stained cells were acquired on a BD Biosciences FACS Aria III using FACS Diva software or a CYTEK Aurora using SpectroFlo software. Acquired flow data were analyzed with FlowJo software v.10.6 or v.10.7 (Tree Star).

T cell-keratinocyte co-culture experiments

T cells were sorted according to the cTRM (CD4 $^{+}$ /CD8 $^{+}$ and CLA $^{+}$ CD103 $^{+}$) or T_{CONV} (CD4 $^{+}$ /CD8 $^{+}$ and CLA $^{-}$ CD103 $^{-}$) phenotype and rested overnight in RPMI 1640 (Gibco; 52400025) with 10% FBS (Gibco; 10500064) and 1% penicillin-streptomycin (Gibco; 15140122). Keratinocytes isolated from healthy donor skin by explant cultures were seeded in 8-well chamber slides (ibidi; 80897) at 20,000 cells per well. T cells were stimulated for 2 h with phorbol 12-myristate 13-acetate (Sigma-Aldrich; P1581-1MG) at 10 ng/ml and 1 μ g/ml Ionomycin (Sigma-Aldrich; I0634-1MG), and then 1,000 cTRMs or T_{CONV} cells were added per well

in X-VIVO 15 Medium (Lonza; BE02-060F). Cells were cultured for 16 h before T cells were removed by washing. Keratinocytes were stained with GFP LIVE/DEAD Fixable Green Dead Cell Stain Kit (Invitrogen; L34969) according to the manufacturer's instructions and subsequently fixed with 4% formaldehyde. Afterward, CD3-AF750, Bcl-2-PE, and purified TSLP with a second-step AF647 were stained as previously described (see Table S1 for antibody details; Brüggen et al., 2014). Briefly, cells were permeabilized with 2% BSA + 0.1% Triton-X-100 and subsequently stained with unlabeled, second-step, and labeled antibodies for 1 h each at 37°C and 5 min with DAPI at room temperature. Mounted slides were imaged on a Zi Axio Observer microscope equipped with an LD Plan-Neofluar 20×/0.4 objective (Zeiss) and TissueFAXS imaging system and analyzed using TissueQuest software (TissueGnostics GmbH). Data from sorted CD4⁺ and CD8⁺ T cells were combined for analysis.

cTRM staining of GI-GVHD biopsies

Cryosection of optimal cutting temperature media-embedded gut biopsies from patients with acute GI-GVHD were acetone-fixed and stained for cTRM markers CD3, CD4, CLA, and CD103 as previously described (Brüggen et al., 2014; antibodies listed in Table S1). Briefly, after blocking with rat and mouse serum (+2% BSA), the tissue was stained with fluorochrome-conjugated antibodies for 2 h, Alexa Fluor 488-labeled anti-FITC for 30 min, and DAPI nuclear marker for 5 min. All steps were performed at room temperature. Slides were imaged as described above.

Cytospins and X/Y fluorescence in situ hybridization of T cell subsets

T cells were sorted in Dulbecco's PBS with 10% FBS. Cytospins of >1,000 cells were harvested onto microscope slides using a cytofunnel system (Shandon) and processed using FAST FISH Prenatal X, Y and 18 Enumeration Probe Kit (Cytocell; LPF 002) as described previously (Strobl et al., 2020b). In brief, the protocol included a digestion step with pepsin/HCl, fixation with formaldehyde, denaturation with formamide, hybridization at 81°C, incubation with the fluorescence in situ hybridization (FISH) probe overnight at 37°C, and DAPI staining before mounting. Slides were imaged as described above.

Smart-seq2 bulk and single-cell RNA-seq

For Smart-seq2 (Picelli et al., 2014), bulk (Fig. 1, A and B; Fig. 2, E–H; and Fig. 6 G) and single-cell (Fig. 4) RNA-seq libraries were prepared and sequenced at the Biomedical Sequencing Facility of the Center for Molecular Medicine of the Austrian Academy of Sciences as previously described (Strobl et al., 2020b). Bioinformatical analysis, including gene expression analysis, SNP calling, and T cell clonality analysis, was performed as previously described (Strobl et al., 2020b). Determination of single-cell T cell clones from Smart-seq2 sequencing was identified using the TRUST (TCR repertoire utilities for solid tissue) tool version 4.0 (Li et al., 2016). For pathway enrichment (Fig. 6 G), DEGs in cTRMs with adjusted P value <0.05 were loaded in Enrichr (Xie et al., 2021), and the top 25 KEGG 2021 human pathways with adjusted P value <0.01 were plotted.

10X Genomics single-cell sequencing

For 10X Genomics single-cell sequencing, CD45⁺ live cells were sorted into PBS/10% FCS for 10X library preparation with the Single-Cell 3' Library & Gel Bead Kit v2 (10X Genomics) according to the manufacturer's instructions. Briefly, sorted cells were partitioned into gel bead-in-emulsions for cDNA synthesis and subsequently amplified and prepared for sequencing. The samples were sequenced at the Biomedical Sequencing Facility on the Illumina HiSeq 3000/4000 platform in the 75-bp paired-end configuration. Raw sequencing data were processed and analyzed using the 10X Genomics Cell Ranger platform and 10X Genomics Loupe Browser.

Mathematical modeling of cTRM origin

Whole T cell numbers and TRM numbers were determined per cubic centimeter of skin as described by Clark et al. (2006) and per milliliter of blood at B and Tx and extrapolated to whole-body surface and whole blood volume for an average human adult (weight: ≥66.1 kg) using the Du Bois formula ($BSA = 0.007184 \times W^{0.425} \times H^{0.725}$, Fig. 3, A–C). T cell cycles (Fig. 3 D) were modeled using a system of ordinary differential equations based on previously published models of T cell dynamics in HIV infection (Reeves et al., 2017). The equation system was modeled in Jupyter Notebook software (Python). Plots were created using Matplotlib visualization tool (Hunter, 2007).

Statistical analysis

Statistical analyses on flow cytometry and imaging data were performed using GraphPad Prism 8.0. To determine statistically significant differences between two groups, we used paired or unpaired, two-tailed Student's *t* test. To compare multiple groups, we used parametric one-way ANOVA followed by Tukey's pairwise comparisons and two-tailed Student's *t* test.

Online supplemental material

Fig. S1 shows graphical representation of the patient cohorts. Fig. S2 shows the RNA-based progression score of ITGA1 expression in patient T cells, gating/sorting strategy and FACS-based quantification of T_{naïve}, T_{CM}, T_{EM}, and T_{EMRA} cells, and quantification cross-shared TCR clones in pre-Tx skin versus post-Tx blood. Fig. S3 shows quantification of total PBMC and cTRM numbers in blood of patients at B and Tx and modeled growth curves for scenarios 3 and 4, as well as tested proliferation rates for scenarios 1–3. Fig. S4 shows a t-SNE plot of Smart-seq2 single T cells of patient 2 at d 14 and uniform manifold approximation and projections (UMAPs) depicting expression of TCR genes in GVHD 10X Genomics single-cell data. Fig. S5 shows negative control for IL-13 staining on cTRMs, heat map of selected DEGs in bulk sequenced cTRMs versus T_{CONV} cells of GVHD patients, flow cytometry quantification of α4β7 expression on cTRMs and T_{CONV} cells, and selected markers on CD8⁺ T cells in GVHD blood. Table S1 lists antibodies used in the study. Table S2 and Table S3 show host chimerism of blood T cells in patients A and B, corresponding to Fig. 4. Table S4 lists cross-shared TCR clone sequences of pre-Tx skin and post-Tx blood per patient. Table S5 shows patient details, including clinical information.

Data availability

Sequencing data presented in Fig. 1 B and Fig. S2 A are accessible in Gene Expression Omnibus under accession no. [GSE146495](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146495). All remaining sequencing data are available via Gene Expression Omnibus database accession no. [GSE183212](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE183212).

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Author contributions: J. Strobl and G. Stary designed the study. J. Strobl, L.M. Gail, and L. Kleissl planned and carried out experiments and acquired data. R. Dingelmaier-Hovorka, D. Atzmüller, and T. Krausgruber performed experiments and acquired data. J. Strobl, L.M. Gail, R.V. Pinay, and V. Puxkandl analyzed, interpreted, and visualized the data. V. Smejkal, J. Huber, and J. Strobl designed, analyzed, and visualized the mathematical model. L. Unterhuggauer, P. Wohlfarth, and W. Rabitsch provided patient material and performed disease scoring. J. Strobl and L.M. Gail compiled the figures. J. Strobl, L.M. Gail, and G. Stary drafted the manuscript. C. Bock provided resources and critically revised the manuscript. G. Stary provided funding and resources and supervised the study. All authors reviewed the final version of the manuscript.

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Supplemental material

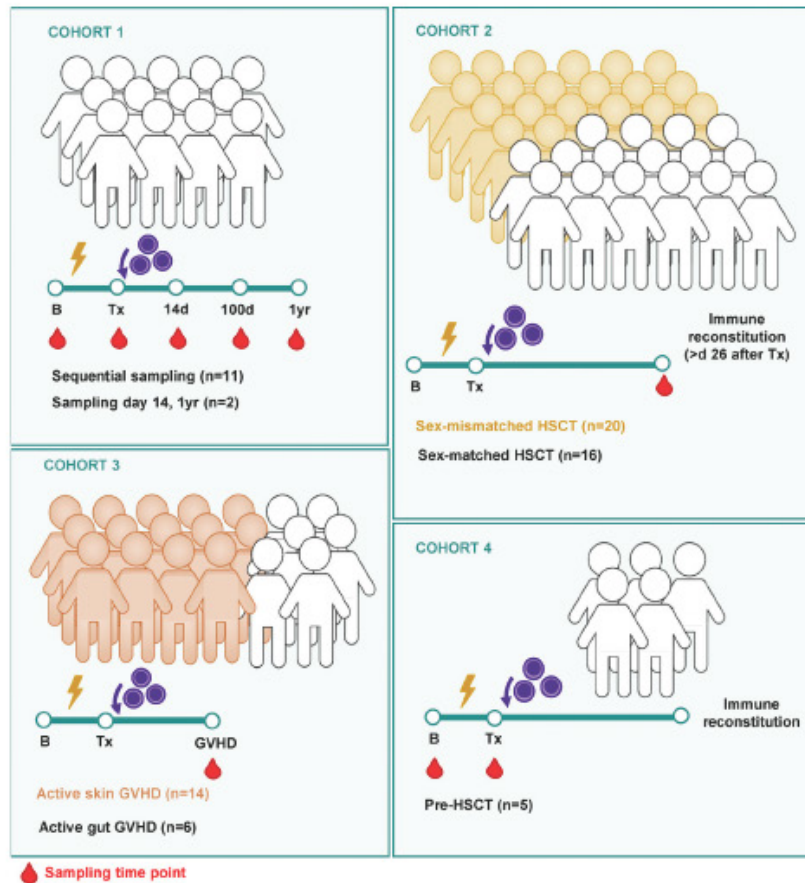


Figure S1. Graphical representation of patient cohorts.

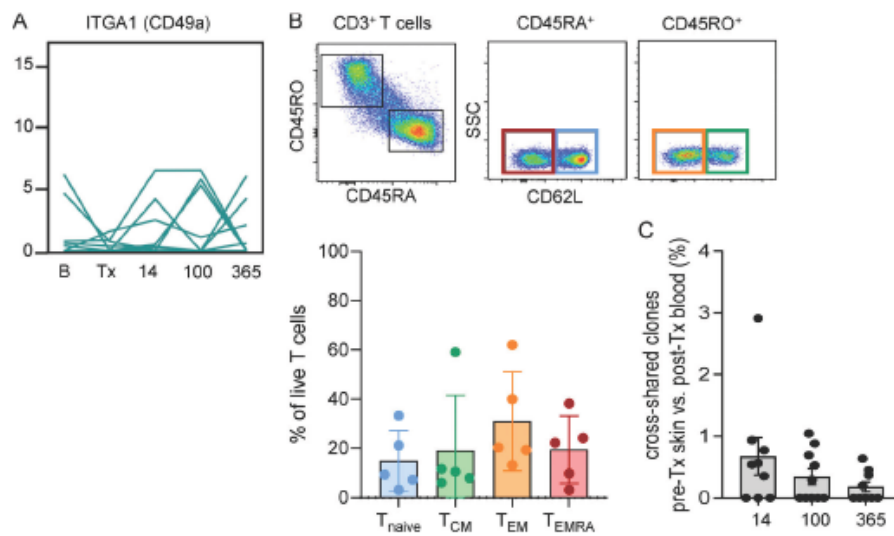


Figure S2. RNA expression, T cell subtypes and cross-shared clones. (A) RNA-based progression scores of *ITGA1* in FACS-sorted bulk T cells. Data shown as mean expression in 100 T cells per patient per time point ($n = 11$). (B) Representative sorting strategy of T cell subsets (T_{naive} = blue, T_{CM} = green, T_{EM} = orange, T_{EMRA} = red) for XY-FISH (Fig. 2C), pregated on live single CD3⁺ cells, and quantification ($n = 5$). Data shown as percentage $\pm$ SEM of live CD3⁺ T cells. (C) Cross-shared clones of sorted T cells from pre-Tx skin and post-Tx blood at days 14, 100, and 365 after transplantation as identified by Smart-seq2/TRUST pipeline. Data shown as mean percentage $\pm$ SEM of all detected TCR clones in each sample ($n = 11$ patients). SSC, side scatter.

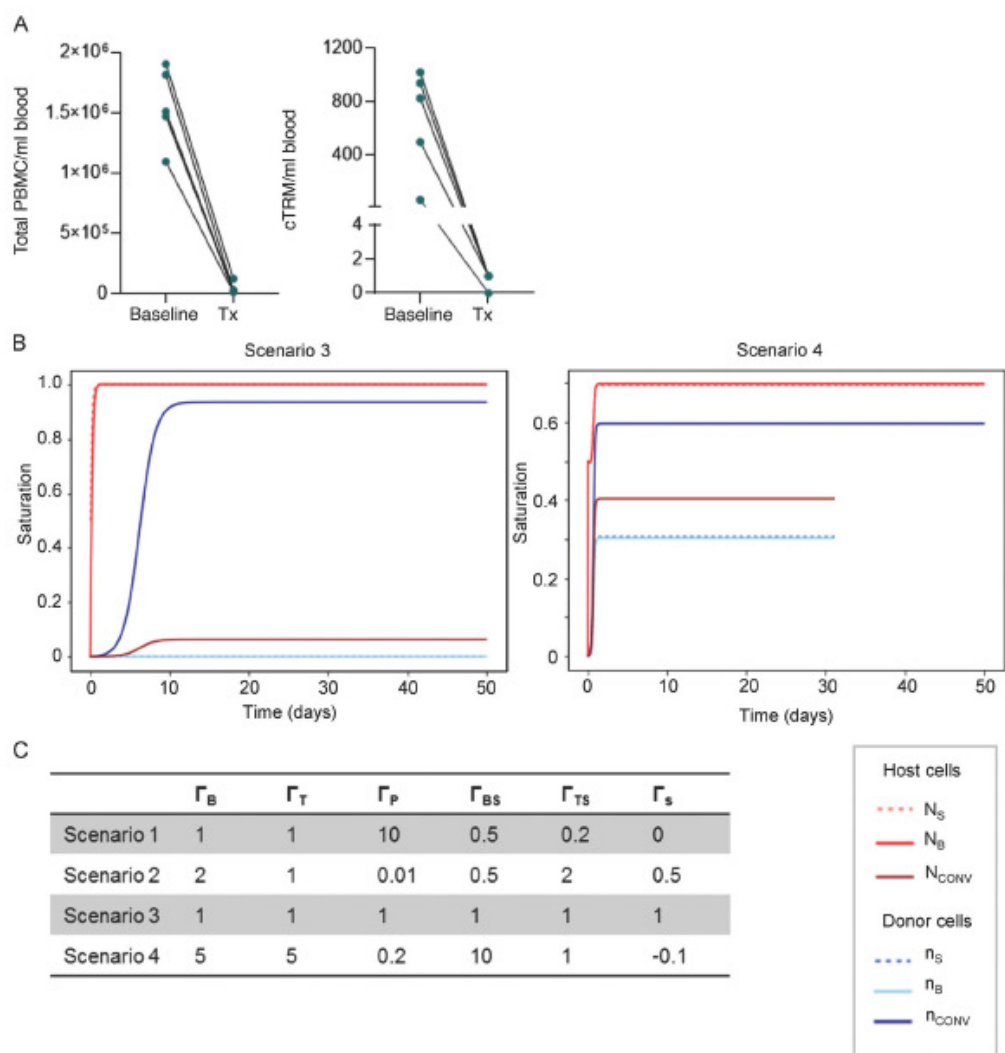


Figure S3. **Mathematical model.** (A) Quantification of total blood PBMCs (left) and total number of cTRMs (right) per milliliter of blood in patients at B and at Tx ($n = 5$). (B) Scenarios 3 and 4 using cell proliferation rates shown in Fig. S3 C. (C) Tested proliferation rates for scenarios 1–4.

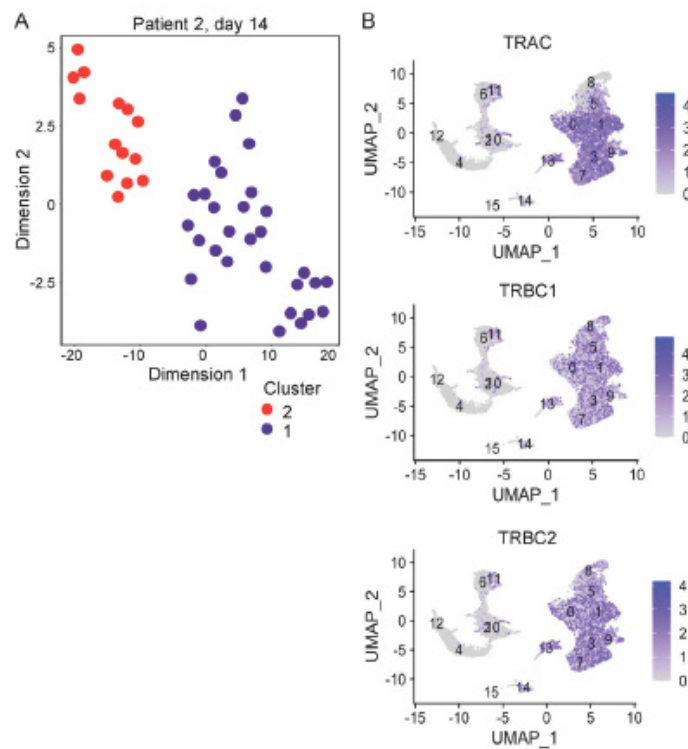


Figure S4. Single-cell RNA expression. (A) Representative t-SNE plot of single blood T cells from patient 2 at day 14 after HSCT as determined by single-cell RNA-seq (Smart-seq2). Unsupervised clustering resulted in two distinct clusters. One dot represents one T cell. (B) UMAP feature plots of log₂ gene expression of TCR α and β genes (TRAC, TRBC1, and TRBC2) in blood cells in GVHD determined by single-cell RNA-seq (10X Genomics; $n = 2$). Cluster 13 represents cells with cTRM phenotype.

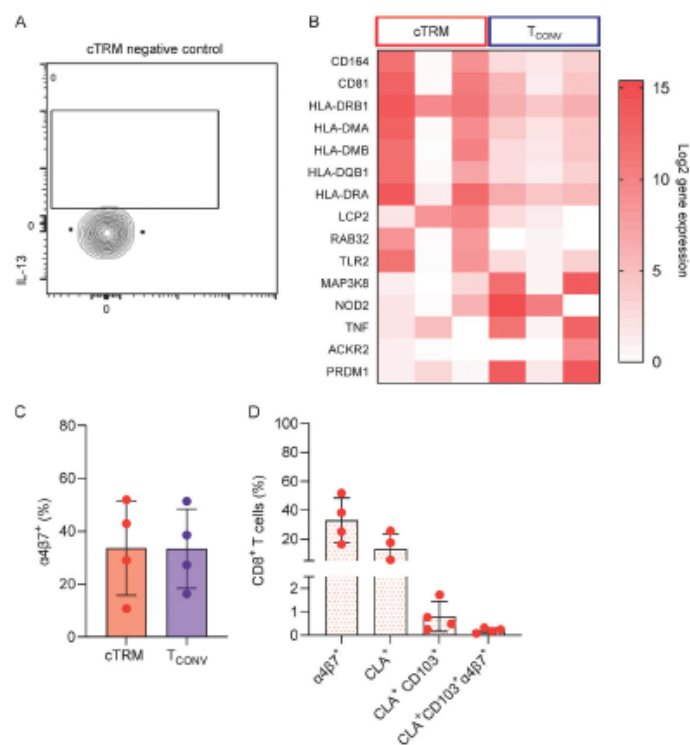


Figure S5. **Additional flow cytometry data and differential gene expression.** (A) Matched negative control of IL-13 expression from cTRMs to plots in Fig. 6 D. (B) Heat map showing log₂ gene expression of selected DEGs (adjusted P value <0.05) comparing bulk transcriptomes of sorted cTRMs and T_{conv} cells (n = 3). (C and D) Mean percentage (±SEM) of α4β7-integrin-expressing CD8⁺ cTRMs and T_{conv} (C) and of CD8⁺ T cells positive for the indicated markers in the blood of GVHD patients (n = 4; D) determined by flow cytometry.

Table S1 lists antibodies and dyes used for flow cytometry, FACS sorting, and immunofluorescence. Table S2 presents host chimerism in peripheral blood CD3⁺ cells of patient A. Table S3 presents host chimerism in peripheral blood CD3⁺ cells of patient B. Table S4 lists shared TCR clone sequences in post-Tx blood and pre-Tx skin for every patient. Table S5 presents patient details.

2.2. Interlude

In the publication in section 2.1, we described the recirculation potential of human skin TRM and discovered that this process seems to be regulated by the tissue immune state, as the number of host-derived cTRM is increasing during ongoing inflammation. However, whether distinct TRM subsets are present in the skin at certain timepoints after HSCT, as well as general processes regulating TRM development are still poorly understood. Therefore, we next set out to investigate TRM cell fate and functional heterogeneity within the tissue. As before, we harnessed the power of longitudinal sampling of patients after HSCT and during active cutaneous aGVHD. In the following study, we generated a time-resolved high-dimensional single-cell transcriptome and TCR dataset of CD45⁺ immune cells of blood and skin, as well as CD45⁻ cells of the skin. We performed diverse bioinformatic analysis and validation experiments to I) track donor-derived T cell clones from blood to skin, II) characterize the functional heterogeneity of host and donor TRM and III) define subsets that are dysfunctional in aGVHD-related inflammation. We found that donor TEM give rise to TRM after HSCT and that a specific subset of type 17 polarized CCR6⁺ CD127⁺ TRM (TRM17) is decreased and dysfunctional in lesional aGVHD skin. TRM17 seem to be impaired both developmentally and in maintenance by the type 1 inflammatory environment that characterizes alloreactive immune responses in cutaneous aGVHD. The graphical abstract illustrating the main findings of this manuscript is shown in Figure 5.

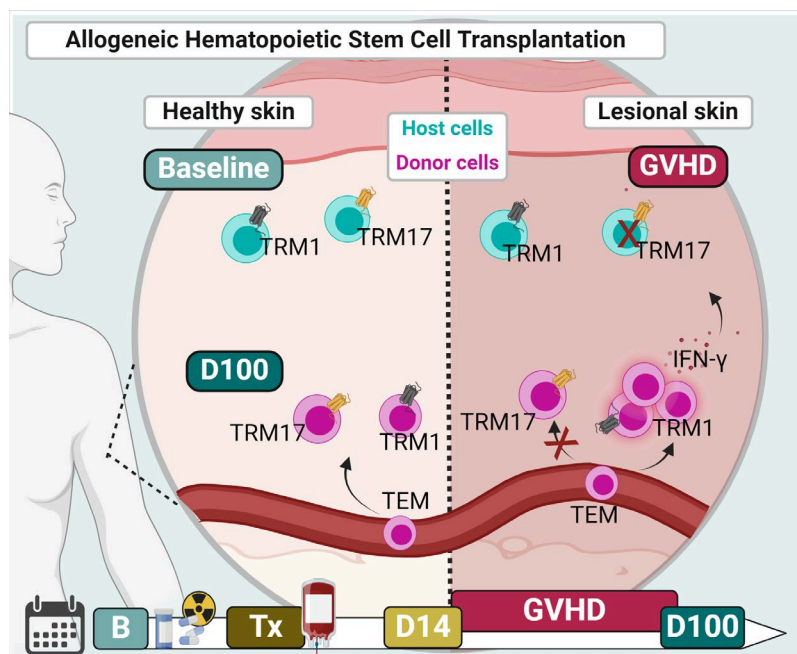


Figure 5 – Graphical abstract for the manuscript “Human type 17 tissue-resident memory T cells are dysfunctional in type 1-driven skin inflammation”

2.2.1. Human type 17 tissue-resident memory T cells are dysfunctional in type 1-driven skin inflammation

TITLE: Human type 17 tissue-resident memory T cells are dysfunctional in type 1-driven skin inflammation

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ABSTRACT

Tissue-resident memory T cells (TRM) maintain homeostasis while also driving inflammatory conditions but underlying regulatory dynamics in humans are poorly understood. Here, we investigate human skin TRM biology in a unique clinical setting: patients undergoing allogeneic hematopoietic stem cell transplantation, with or without graft-versus-host disease (GVHD). Using single-cell RNA and TCR sequencing, we comprehensively explore TRM dynamics, uncovering their transformations in steady-state and inflammation. We identify donor effector memory T cells as primary source for skin TRM post-transplantation. While various TRM subsets co-exist in balance in homeostasis, type 17-polarized TRM (TRM17) are sharply reduced and dysfunctional in IFN- γ -primed GVHD lesions yet recover upon resolution of inflammation. These insights highlight the adaptive plasticity of human skin TRM and propose their balance as prerequisite for tissue homeostasis.

INTRODUCTION

Human skin is rich in immune cells with tissue-resident memory T cells (TRM) playing a crucial role in regulating physiological processes to maintain tissue homeostasis¹⁻³. However, TRM also contribute to inflammation and are often implicated in the pathogenesis of skin diseases⁴⁻⁶, including acute cutaneous graft-versus-host disease (hereafter referred to as GVHD)⁷⁻⁹. GVHD is a T cell-mediated inflammatory disease occurring after allogeneic hematopoietic stem cell transplantation (HSCT), usually manifesting 14-100 days after transplantation, with skin as a main target organ¹⁰. GVHD is mainly induced by type 1-polarized, alloreactive donor T cells, causing tissue damage in target tissues¹⁰. Our laboratory, along with others, has previously demonstrated the persistence of host TRM in the skin after myeloablative conditioning of HSCT patients^{8,9,11,12}. Following donor cell engraftment, host TRM are not fully replaced by donor TRM but persist for at least 10 years after conditioning treatment⁸. This makes skin and blood samples from HSCT patients, including those developing GVHD, a valuable resource for studying TRM reconstitution and maintenance in healthy skin, as well as in acute, T cell-mediated inflammation.

Skin TRM are usually identified by the expression of CD69 and CD103, but recent studies have revealed developmental and functional plasticity within this population. In mice, most studies have focused on type 1-polarized TRM (TRM1), which are generated in response to viral infection and depend on transcription factors Hobit and T-bet^{13,14}. In murine skin, however, so-called TRM17 are developmentally independent from TRM1¹⁴. TRM17 express CCR6, depend on the transcription factor c-MAF, require IL-7 signaling through CD127, and are generated in response to *Staphylococcus epidermidis* colonization¹⁴. Murine TRM17 are ICOS⁺ and can be selectively depleted by targeting ICOS¹⁴. In human skin, CCR6⁺ TRM17-like cells are enriched in psoriatic patients^{5,15} and are functionally distinct from CD49a⁺ TRM in the epidermis¹⁶. CCR6⁺ T cells are major producers of IL-17A, IL-22, TNF- α and GM-CSF, whereas CD49a⁺ T cells have a TRM1-like phenotype and primarily produce IFN- γ ¹⁶.

So far, it remains unclear whether human TRM subsets are regulated by the same transcriptional programs and have similar survival dynamics as murine TRM subsets. Moreover, there are conflicting reports regarding the formation of TRM from effector memory

T cells (TEM) or central memory T cells (TCM), with evidence for both scenarios in different models^{17,18}.

We aimed to track TRM reconstitution and assess TRM subset heterogeneity in homeostasis and inflammation in human skin, leveraging skin and blood samples from recipients of HSCT, developing, or not developing GVHD. To assess the transcriptional profiles and clonal relationships among the various T cell subsets, and examine changes in the tissue microenvironment, we generated a high-dimensional, time-, tissue-, genotype- and clonotype-resolved single-cell RNA and T cell receptor (TCR) sequencing dataset, enabling an in-depth investigation of TRM biology with functional validation.

Our analysis reveals that donor TEM are the main source of skin TRM generation following transplantation. In homeostatic conditions, TRM17 and TRM1 co-exist in balance in the skin. However, in GVHD lesions, the IFN- γ -primed, pro-inflammatory microenvironment is associated with a profound reduction and a disturbed function of TRM17. These findings suggest that exacerbated type 1 inflammation alters the identity, generation, and maintenance of the TRM17 pool. Importantly, after the resolution of inflammation TRM17 are restored to levels seen in healthy skin, underscoring plasticity and adaptability of the TRM pool and suggesting a crucial role for balanced TRM17 and TRM1 responses in maintaining tissue homeostasis.

RESULTS

Single-cell RNA sequencing captures cellular heterogeneity and dynamics of immune chimerism after transplantation

To track developmental and temporal dynamics of cell states at high resolution, we performed single-cell RNA sequencing of skin and blood samples of a HSCT patient cohort at four key timepoints: baseline (before myeloablative conditioning), on the day of transplantation (Tx), and 14 and 100 days post-transplantation (D14, D100) (Fig 1a,b). From seven patients, 21 matched skin and blood samples across all time points were sequenced (Fig 1b, HSCT). We also included blood and lesional skin from four patients developing GVHD after HSCT from

our published dataset for comparison¹¹, which were only sampled at disease onset (Fig 1a,b, GVHD). One patient (P6) was part of both the HSCT and the GVHD cohort. The detailed clinical characteristics of patients included in the study are listed in Table S1.

After quality control and filtering we recovered a total of 60,019 high-quality cells with broad representation of all patient samples (Fig 1c, Fig S1a), clearly segregating into skin and blood specific cell states (Fig 1d). Myeloid, lymphoid and non-hematopoietic cells were further subclustered and annotated separately within each group (Fig S1b-e, Fig 2a,b). We then used this high-resolution cluster information to recalculate the uniform manifold approximation and projection (UMAP) and to condense the subclusters to broad immune and non-hematopoietic cell type classes characterized by the expression of canonical makers (Fig 1e,f). Within CD45⁺ lymphocytes, we detected 9 T cell and 2 natural killer (NK) cell clusters, which are described in greater detail below (Fig 2a,b). Among myeloid cells, we detected three distinct monocyte clusters in the blood (Mono), which corresponded to classical, intermediate and non-classical monocytes (Fig S1b,c). Dendritic cells (DC) segregated into classical DC subtypes (cDC1, cDC2, cDC_{mig}), and we detected Langerhans cells (LC) and plasmacytoid DC (Fig S1b,c). Skin macrophages (MΦ) clustered into three groups, characterized by activated and pro-inflammatory signatures (MΦ, MΦ_{Act}, MΦ_{Inf}), (Fig S1b,c). Non-hematopoietic skin cells comprised 6 clusters of dermal fibroblasts (Fibro), including three reticular fibroblasts (F_{ret} ECM, F_{ret} act, F_{ret} im) defined by extracellular matrix production, activation and immunomodulation, respectively (Fig S1d,e). Papillary fibroblasts (F_{pap} ECM) expressed papillary markers, while two smaller clusters were associated with hair follicles: dermal sheath fibroblasts (F_{DS}) and dermal papilla fibroblasts (F_{DP}). We also detected vascular and lymphatic endothelial cells (vEC/IEC), alongside two pericyte (Pc conv, Pc im) clusters (Fig S1d,e). Epidermal cells comprised basal keratinocytes (KC_b) and suprabasal keratinocytes (KC_{sb}) as well as melanocytes (Mlc), (Fig S1d,e). Detailed markers used for myeloid and non-hematopoietic subcluster annotation are provided in Fig S1c and Fig S1e, respectively. Collectively, we generated the first time-resolved HSCT single-cell RNA and TCR sequencing dataset, comprising the main immune and non-hematopoietic cell types of human blood and skin.

By genetic definition of donor and host cells through single nucleotide polymorphisms (SNPs) or gender mismatch^{8,12}, we can track in our unique dataset donor T cell repopulation in the skin and blood, monitor skin TRM over time and study pathogenic mechanisms in GVHD. Here we employed a workflow using SNP-based demultiplexing, aiming to accurately assign the genotype of each cell¹⁹ (Fig 1g).

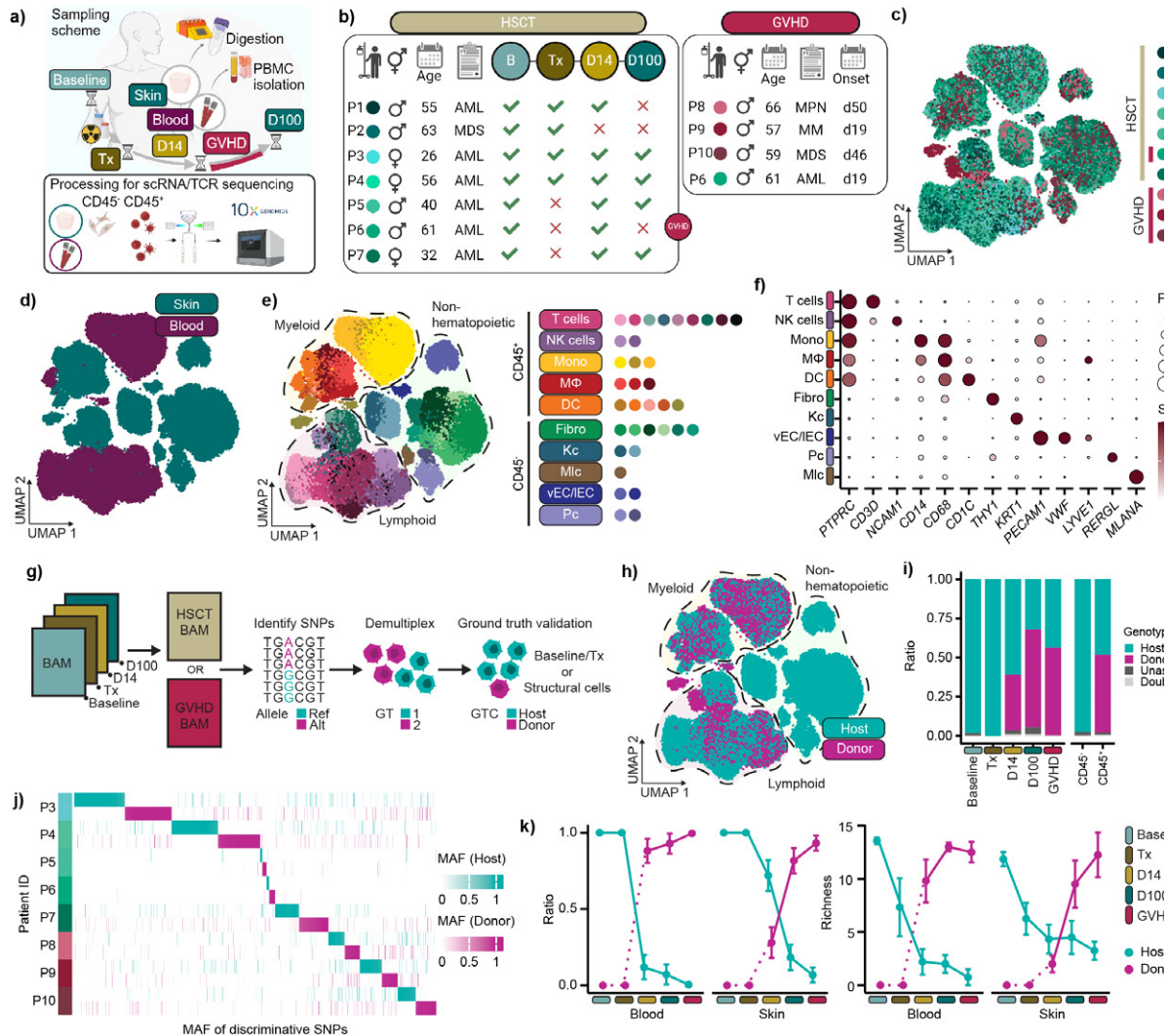


Figure 1—Single-cell RNA sequencing analysis of a time-, tissue-, cell type- and genotype-resolved dataset during HSCT and GVHD

a) Scheme of the sampling and sorting strategy for single-cell transcriptomic profiling. b) Overview of the patient cohort. c-e) UMAP visualization of all cells recovered from single-cell RNA sequencing, colored by Patient ID (c), tissue source (d) and cell types identified after Leiden clustering (e), dots assigned to each cell group represent Leiden clusters. f) Dot plot of marker gene expression used to annotate cell type classes. g) Scheme depicting the strategy for single nucleotide polymorphism (SNP)-based host/donor genotype demultiplexing. h) UMAP of all cells colored by host/donor genotype annotation. i) Bar chart of host/donor demultiplexing results across time points or in CD45⁺ vs CD45⁻ cells. j) Heatmap of minor allele frequency (MAF) of discriminative host or donor SNPs in each patient used for demultiplexing. k) Host/donor ratio (left) and richness, representing the number of detected cell types (right), in all CD45⁺ immune cells in skin and blood over time points.

We successfully distinguished host and donor cells in all patients with clinical donor engraftment post-transplantation. P1 experienced graft failure and passed away at d38, and patient P2 dropped out of the study after Tx (Fig 1b), hence no donor cells were detected. Few cells with uncertain assignment (doublets (0.36%) or unassigned (1.65%)) were removed from subsequent analysis. Of the remaining cells less than 0.01% in baseline samples and of non-hematopoietic cells were falsely classified as donor, proving high confidence genotyping (Fig 1h,i). In each host/donor pair we identified a set of unique discriminative SNPs (Fig 1j). Following immune reconstitution, we observed the anticipated rapid dynamic switch from host to donor immune cells in the blood, whereas this replacement was slower and incomplete in skin (Fig 1k). Notably, the skin exhibited greater host cell diversity compared to blood, reflecting a more complex immune cell turnover in the skin (Fig 1k).

On D14 and D100 an average of $71.98\% \pm 10.05\%$ (SEM) and $18.30\% \pm 8.33\%$ (SEM) of skin immune cells were host-derived, respectively. In GVHD, the mean contribution of host cells was $6.83\% \pm 4.95\%$ (SEM) (Fig 1k). This contrasts with our earlier findings of $>50\%$ host T cells in certain GVHD patients yet aligns with the considerable variability in host chimerism observed by our group and others^{8,12}.

In summary, SNP-based demultiplexing is a robust method to accurately annotate host and donor genotypes in single-cell RNA sequencing data, allowing us to separately track host and donor immune reconstitution and maintenance dynamics in blood and skin of HSCT patients.

Donor effector T cells generate new tissue-resident T cells following transplantation

We next investigated clonal and transcriptional relationships of blood and skin T cells to track donor TRM formation by transplanted donor T cells. As previously noted, we identified nine distinct T cell states after sub-clustering of lymphocytes (Fig 1e, Fig 2a,b). T cell clusters were categorized based on *CD4* and *CD8A/CD8B* expression levels. Within each group there was a central memory/naïve T cell cluster (TCM/N: *TCF7*, *CCR7*, *SELL*) and a *CCR7/SELL*^{low}, *CD44*-positive effector/memory T cell cluster (TEM) in blood. A corresponding TRM cluster (*CD69*, *PRDM1*, *CREM*) was identified in the skin. CD4 T regulatory cells (Treg) expressing *FOXP3* formed a separate cluster and were spatially distinguished by their affiliation to either

blood or skin (Fig 2b). Additionally, a cluster of invariant T cells (*Tinv*) was characterized by *CD3D* expression, but absence of *CD4* or *CD8A/CD8B* transcripts, and a cluster of cycling T cells (Cycling) displayed high *MKI67* expression (Fig 2b). NK cells were divided into *CD16⁻* and *CD16⁺* NK cells, displaying high and low expression levels of *NCAM* and the opposite expression patterns for *FCGR3A*, respectively.

Integration of TCR- α/β sequencing data revealed a notable overlap with the annotated T cell clusters, and highlighting areas of significant clonal expansion within CD8 TEM, CD8 TRM and cycling cells (Fig 2c). We successfully recovered T cell clones across all timepoints from all T cell clusters in both tissues (Fig 2d). Donor-derived immune cells were detected in the skin as early as D14 (Fig 1k). Consequently, we sought to further explore the formation of TRM from donor T cells of different blood clusters.

We extracted all donor-derived T cells that shared clones in blood and skin from either D14 and D100 (HSCT), where T cell recruitment occurs in the absence of inflammation, or from GVHD samples, where skin is inflamed. When plotting the cluster affiliation of these shared clones in both HSCT and GVHD, we observed a strong overlap between blood TEM and skin TRM, as well as cycling cells, indicating ongoing TRM generation by donor TEM cells. In GVHD, there was limited overlap of CD4 TCM/N and CD4 TRM (Fig 2e).

Next, we conducted a differential gene expression comparing shared clones from blood and skin and observed upregulation of residency regulators in both HSCT and GVHD (*CD69*, *RUNX3*, *FABP5*, *BHLHE40*, *NR4A2*, *CXCR4*)^{8,20-24} (Fig 2f). To assess whether the majority of T cells in the skin clusters represented true TRM and upregulation of a resident phenotype occurred across all donor skin T cells, we computed a module score (MS) for residence and recirculation based on canonical genes that distinguish these phenotypes. We then calculated the MS difference for all CD4 or CD8 blood TEM and skin TRM cells (Fig 2g). Donor skin T cells had high differential MS values, indicating residence, except for a subset of donor cells in the CD8 TRM cluster in GVHD with negative scores, indicating recirculation, in line with effector or central memory T cell infiltration during inflammation (Fig 2g). Overall, our data supports the notion that donor-derived TEM are the primary source for TRM formation in the skin, although a contribution from TCM/N during inflammation may also be possible.

while fewer genes were differentially expressed on Tx, D14, and D100 (Fig 3a). The top

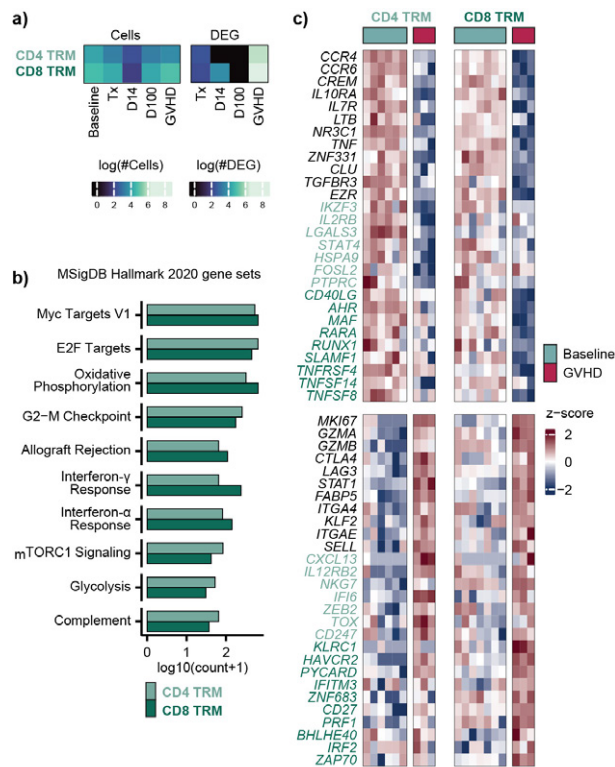


Figure 3 – Differential gene expression analysis of skin TRM before transplantation and upon GVHD

a) Heatmap of Cell log count per time point (left) and number of differentially expressed genes (DEG) comparing time points vs Baseline (right) within skin CD4 TRM and CD8 TRM. b) Significantly enriched Hallmark 2020 gene sets from genes upregulated in GVHD in CD4 TRM and CD8 TRM; adjusted p-value cutoff for pathway enrichment <0.05. c) Heatmap of gene expression of selected DEG at Baseline or GVHD in CD4 TRM (light green), CD8 TRM (dark green) or both (black), adjusted p-value cutoff for DEG <0.05.

pathway enrichment hits of genes upregulated in GVHD were shared by both CD4 and CD8 TRM (Fig 3b), reflecting pathways related to proliferation and DNA replication (e.g., Myc targets: *TYMS*, *MCM7*, *PCNA*; E2F targets: *DNMT1*, *MKI67*, *TOP2A*; G2-M checkpoint: *CDK1*, *KIF22*), increased metabolic activity (e.g., oxidative phosphorylation: *SCL25A5*, *ATP5F1A*, *COX5A*; glycolysis: *TPI1*, *PGAM1*, *MHD2*) and inflammation (e.g., allograft rejection: *CD74*, *HLA-DRA*, *KLRD1*; interferon- α and - γ response: *BST2*, *ISG20*, *LY6E*, *STAT1*, *LCP2*, *GPB2*, *IRF2*; complement: *CTSD*, *GNAI2*).

Additionally, many genes related to T cell residency, polarization and function were differentially expressed at baseline and in GVHD (Fig 3c). TRM-associated genes

such as *CCR4*, *TGFBR3* and *CREM*^{8,24,25} were downregulated, while genes related to circulatory T cells, like *SELL* and *KLF2*²¹ were upregulated in GVHD, suggesting skin infiltration of circulating effector T cells during GVHD. This is in line with the reduced residency MS in GVHD shown in Fig 2g. In GVHD, T cells displayed elevated expression of cytotoxicity markers (*GZMA*, *GZMB*, *PRF1*), immune checkpoints (*CTLA4*, *LAG3*) and exhaustion markers (*TOX*, *HAVCR2*), while expression of genes associated with regulatory functions (*LTB*, *IL10RA*, *TNF*, *TGFBR3*) was reduced. Notably, *CCR6* and *IL7R*, markers of TRM17, were among the most downregulated genes in both CD4 TRM and CD8 TRM during GVHD (Fig 3c), hinting at a specific impairment of TRM17 cells.

In CD8 TRM from GVHD patients, we observed significant downregulation of *MAF* (encoding for c-MAF) and type 17-related genes, including *AHR* and *RUNX1*^{26,27}. Conversely, we observed upregulation of type 1 TRM master regulator *ZNF683* (the human homologue of Hobit), canonical type 1 genes and upstream signals for T-bet activation (*IL12RB2*, *STAT1*), along with upregulation of TRM related genes *BHLHE40*, *FABP5* and *ITGAE* (Fig 3c).

These transcriptional profiles suggest that in GVHD skin, there is not only a general shift from a tissue-resident, homeostatic signature to an effector cell-infiltrated, cytotoxic one, but also an imbalance of TRM17 and TRM1 cells, particularly within CD8 TRM.

CCR6/IL7R-expressing TRM17 represent a distinct subset and are reduced in GVHD

While TRM17-like cells have been described in human skin, it remains unclear whether they employ the same developmental and maintenance mechanisms as TRM1. The next goal was to better resolve CD8 T cell subsets and assess whether TRM17 form a discrete cluster with distinct temporal and proportional dynamics in human skin. Selecting CD8 TCM/N, CD8 TEM and CD8 TRM and performing partition-based graph abstraction (PAGA²⁸) clustering on skin and blood CD8 T cells revealed four distinct clusters in the skin (Fig 4a). Cluster 4 exhibited high expression of *CCR6* and *IL7R* expression (*CCR6/IL7R^{hi}*), consistent with TRM17 cells (Fig 4a). Cluster 2 showed an elevated expression of the TRM marker *CD69*, Cluster 8 expressed cytotoxic markers *GZMB* and *PRF1*, while Cluster 1 resembled circulating cells with *CCR7* expression (Fig S2a). However, Cluster 2, 8 and 1 were low in *CCR6* and *IL7R* (Fig 4a, Fig S2a), hence we combined these three clusters into a *CCR6/IL7R*-low (*CCR6/IL7R^{lo}*) group.

Interestingly, density maps of CD8 T cells over time revealed that the UMAP region occupied by *CCR6/IL7R^{hi}* cells was notably diminished in GVHD (Fig 4b). Quantifying these cells over time confirmed a clear reduction of the *CCR6/IL7R^{hi}* subset in GVHD (Fig 4c). To check whether *CCR6/IL7R^{hi}* cells share additional transcriptional characteristics with the developmentally independent TRM17 lineage recently described in mice, we performed differential gene expression analysis comparing *CCR6/IL7R^{hi}* and *CCR6/IL7R^{lo}* cells at baseline (Fig 4d). Importantly, *CCR6/IL7R^{hi}* TRM expressed significantly higher levels of *MAF*,

the key regulator of murine TRM17. Other upregulated genes included type 17-genes *AHR*, *CCR4* and transcription factors *KLF6* and *STAT3* (Fig 4d).

Gene regulatory network analysis revealed enrichment of interferon-regulatory factors (*IRF1*, *IRF7*, *IRF9*) and critical type 1-related transcription factor regulons (*EOMES*, *STAT1*) in *CCR6/IL7R^{lo}* cells (Fig 4e). Of note, *TCF7* (encoding TCF1) was among the top hits (Fig 4e). In mice, loss of Tcf1 allows changes in chromatin accessibility to type 17 effector genes, while limiting access to type 1 loci *Tbx21*, *Eomes* and *Irf4*²⁹. In GVHD models, Tcf1⁺ progenitor T cells are a source for alloreactive cells in target tissues³⁰⁻³². In *CCR6/IL7R^{hi}* cells, top hits included known type 17 regulators, such as AP-1 family transcription factors *BATF*, *ATF3* and *JUND*³³⁻³⁵, and *GATA3*, which is known to regulate GM-CSF expression while limiting IFN- γ production^{36,37} (Fig 4e). Additionally, *PPARG*, a crucial regulator of fatty acid metabolism³⁸, which TRM rely on for survival²³, was also enriched. Interestingly, *KLF6*, which promotes T-bet expression in Th17 cells and supports a Th1 fate switch³⁹, was the top hit in *CCR6/IL7R^{hi}* cells (Fig 4e). This may suggest that gene programs promoting lineage instability were already active at baseline. Although *MAF* was not the strongest hit, it still ranked among the top 30 in *CCR6/IL7R^{hi}*, but not *CCR6/IL7R^{lo}* cells (Fig 4e). Overall, our data suggests that the CD8⁺ TRM17 subset present in human skin is selectively reduced in GVHD-affected skin.

Given the replacement of host cells by donor cells in GVHD, we next assessed chimerism dynamics within these clusters. Notably, donor cells were less prevalent in the *CCR6/IL7R^{hi}* UMAP space (Fig 4f). We compared the numbers of host and donor cells within *CCR6/IL7R^{lo}* and *CCR6/IL7R^{hi}* populations over time, observing that while *CCR6/IL7R^{lo}* donor cells increased by D100 and in GVHD, *CCR6/IL7R^{hi}* donor cells did not (Fig 4g). Conversely, some *CCR6/IL7R^{lo}* host cells persisted, whereas *CCR6/IL7R^{hi}* host cells were barely detectable (Fig 4g). These findings suggest that incoming donor cells fail to acquire the TRM17 phenotype in the skin, and that remaining host TRM17 cells are not maintained. This aligns with recent murine studies indicating that TRM1 have a longer lifespan than TRM17¹⁴.

Although the downregulation of type 17-related genes was more pronounced among CD8 TRM, we found similar dynamics for CD4 T cells (Fig S2b-i). In CD4 T cells, one of three skin clusters was characterized by highest *CCR6* and *IL7R* (Cluster 0) (Fig S2c), although other

clusters also expressed these genes at lower levels. Further transcriptional analysis did not reveal a discrete transcriptional profile suggestive of a separate TRM lineage in CD4 T cells, as seen with CD8 T cells (Fig S2f,g). However, we still observed a reduction of the cluster with the highest *CCR6* and *IL7R* expression over time, as well as similar host/donor dynamics (Fig S2e,f,h).

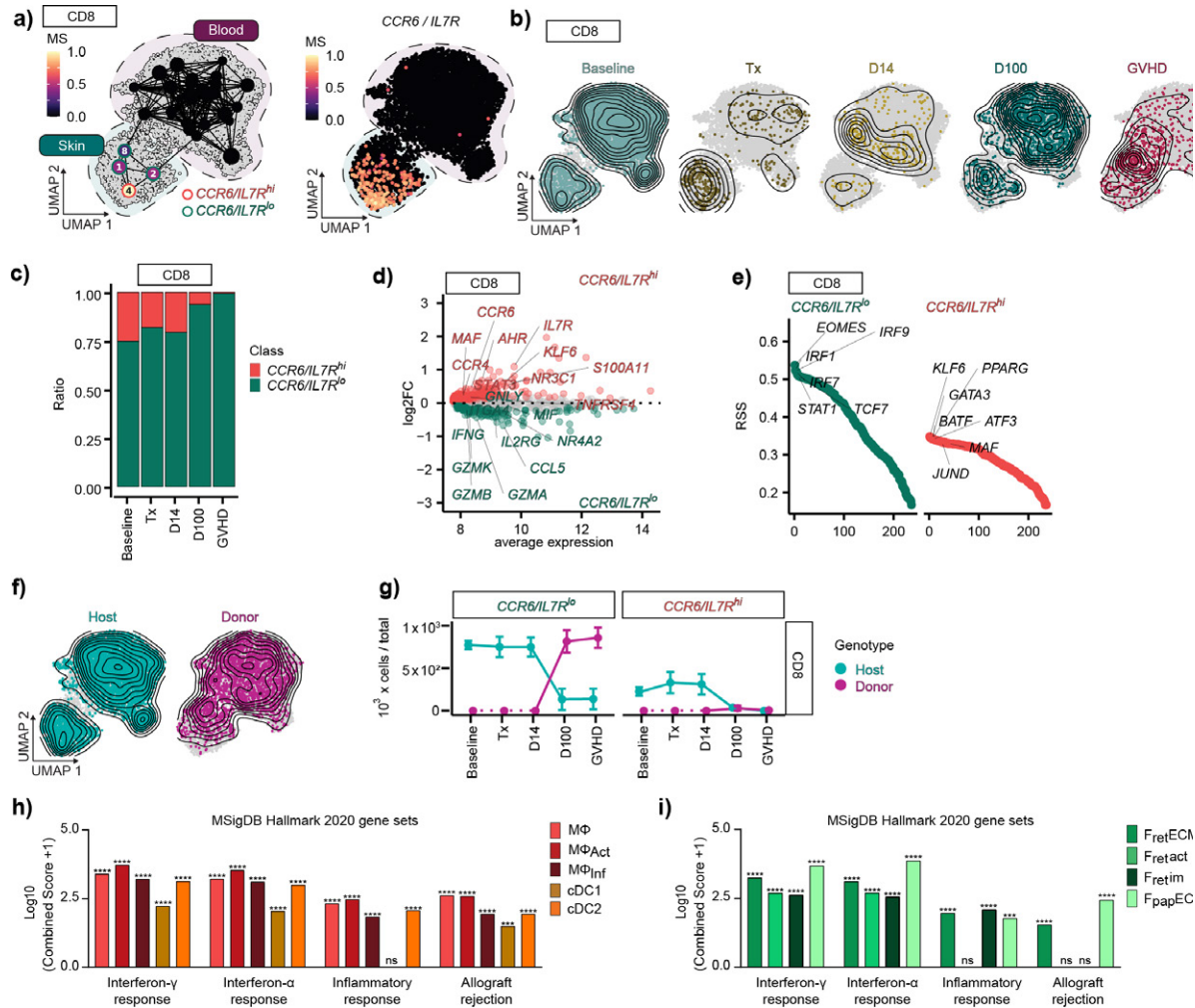


Figure 4 – CD8 T cell sub-clustering and transcriptional analysis to detect TRM17

a) UMAP of all CD8 T cells after re-integration and subclustering of CD8 TCM/N, CD8 TEM and CD8 TRM. Left: Network representing PAGA clustering with clusters as circles, clusters 1,2,4 and 8 represent skin clusters. Cluster filling colored by expression of *CCR6/IL7R* module score (MS) and cluster border colored by annotation to *CCR6/IL7R* *hi* (Cluster 4), or *CCR6/IL7R* *lo* (Cluster 1,2,8) group. Right: Expression of *CCR6/IL7R* MS per cell. b) Density maps on UMAP of CD8 T cells over the tissue sampling timepoints. c) Bar chart of *CCR6/IL7R* *hi* and *CCR6/IL7R* *lo* ratio within all skin CD8 T cells (Clusters 1,2,4,8) over the tissue sampling timepoints. d) Differentially expressed genes in *CCR6/IL7R* *hi* vs *CCR6/IL7R* *lo*, adj p-value cutoff < 0.05. e) Selectively upregulated transcription factors in *CCR6/IL7R* *hi* and *CCR6/IL7R* *lo*. f) Density maps on UMAP of host and donor CD8 T cells. g) Normalized host and donor cell ratios of *CCR6/IL7R* *hi* and *CCR6/IL7R* *lo* host and donor CD8 skin T cells over time. h-i) Combined enrichment scores of selected pathways significantly enriched in genes upregulated in GVHD compared to Baseline across skin myeloid cell clusters (h) and fibroblast clusters (i) (adjusted p-values: ***<0.001, ****<0.0001).

These findings support the existence of an independent lineage of CD8⁺ TRM17 in human skin, which can be identified by co-expression of *CCR6* and *IL7R*. Furthermore, commitment to the TRM17 lineage, as well as its maintenance, appears to be disrupted in GVHD lesions. We suspected that this may be mediated by a strong IFN- γ primed microenvironment, which antagonizes type 17 polarization and the maintenance of type 17-polarized cells⁴⁰⁻⁴². Therefore, we performed pathway enrichment analysis on genes significantly upregulated in GVHD compared to baseline across different myeloid cell and non-hematopoietic clusters in the skin (Fig 4h,i). Of note, the top enriched pathways across macrophages, dendritic cells and fibroblasts included IFN- γ response, IFN- α response, Inflammatory response and allograft rejection (Fig 4h,i), supporting the concept of disturbance of TRM17 in a type 1 inflammatory microenvironment.

TRM17 are reduced and functionally impaired in GVHD

To validate the loss of CCR6- and CD127-expressing CD8⁺ TRM17, and evaluate functional impairments in GVHD on protein level, we collected additional skin biopsies from GVHD lesions and healthy skin as control (HC). Using flow cytometry, we analyzed the expression of TRM markers (CD69, CD103, CCR6, CD127, CD49a, ICOS, CXCR6) and key cytokines (TNF- α , GM-CSF, IL17-A, IL-22 and IFN- γ) (Fig 5a).

We first confirmed that CD8⁺ CCR6⁺ CD127⁺ TRM in healthy human skin display the expected cytokine expression pattern, with expression of TNF- α , GM-CSF, IL-17A and IL-22, but low IFN- γ (Fig S3a,b). Next, unsupervised analysis of all CD8⁺ T cells revealed distinct segregation between healthy and GVHD-derived cells (Fig 5b). Phenograph clustering of TRM surface markers and cytokines identified 11 clusters (Fig 5c), with CI 09 corresponding to TRM17 on the basis of high expression of CD69, CCR6, CD127, TNF- α and GM-CSF (Fig 5d). Differential cluster abundance testing revealed significant enrichment of TRM17 (CI 09) in healthy skin (Fig 5e), as well as another quiescent TRM cluster (CI 10) marked by high CD69 but low cytokine signaling (Fig 5e). Conversely, GVHD skin was enriched in clusters expressing IFN- γ , TNF- α (CI 05) and CD49a (CI 02 and 08), resembling TRM1 (Fig 5 c-e). Interestingly, ICOS expression was not limited to TRM17 in humans, as seen in murine TRM17 cells, but was

broadly expressed in GVHD-enriched clusters, suggesting a potential difference between human and murine skin TRM.

A conventional gating approach on CD8⁺ TRM17 cells confirmed a significant reduction of this subset in GVHD skin lesions (Fig 5f). The remaining TRM17 in GVHD displayed a disturbed functional profile, with decreased TNF- α and GM-CSF production, but increased expression of IFN- γ , CD49a and ICOS (Fig 5g,h). Notably, levels of IL-17A, IL-22, CD69, CD103 and CXCR6 remained unchanged among healthy and GVHD skin CD8⁺ T cells (Fig 5g,h).

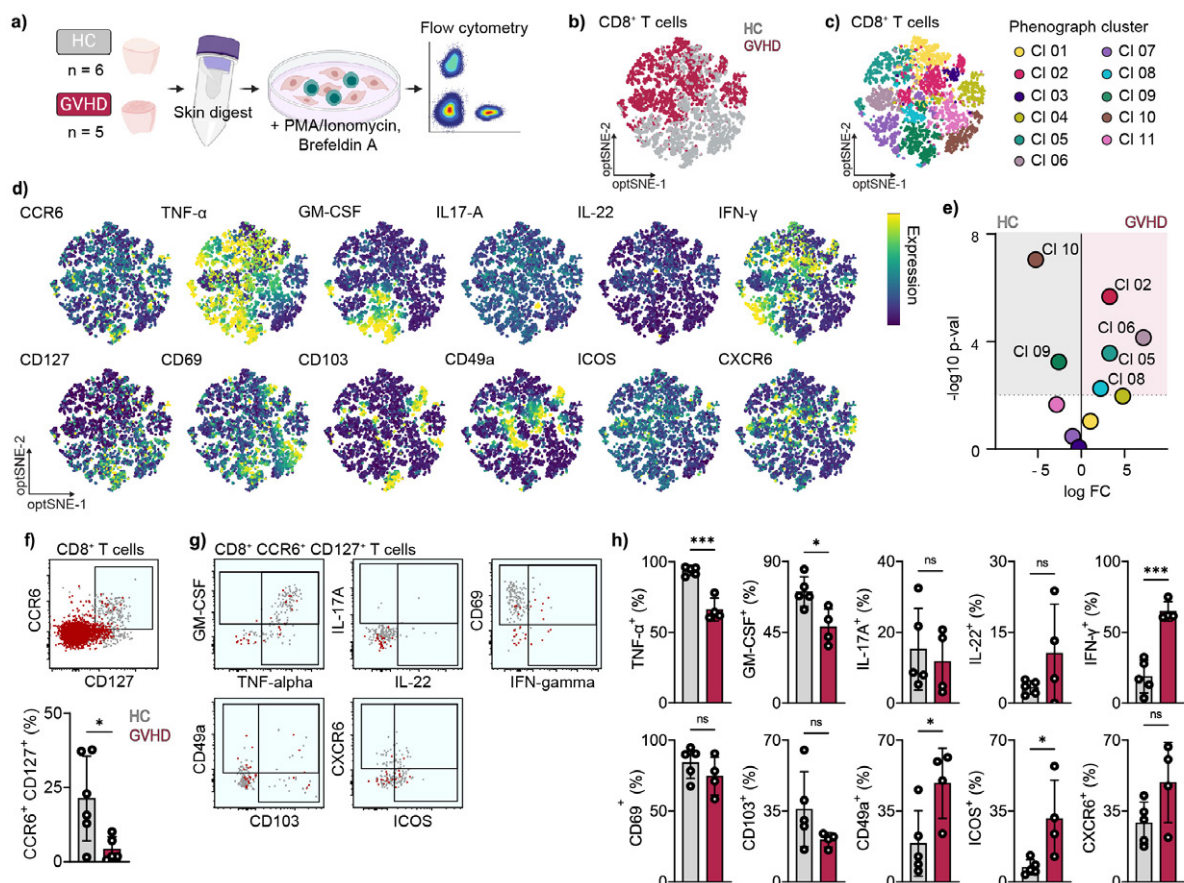


Figure 5 – Phenotypic and functional profiling of CD8⁺ TRM17 in healthy and lesional skin

a) Workflow scheme for T cell profiling in skin biopsies of healthy controls (HC) and lesional GVHD. b-d) Opt-SNE plot of all skin CD8⁺ T cells colored by sample group (b), Phenograph cluster ID (c) or scaled expression of assayed proteins used for calculation of opt-SNE and phenograph clusters (d). e) Volcano plot of differentially abundant clusters in HC or GVHD skin, calculated with EdgeR, p-value cutoff <0.01. f) Representative gating plot and quantification of CCR6⁺ CD127⁺ TRM17 cells among CD8⁺ T cells in HC and GVHD. g-h) Representative gating plots (g) and quantification (h) of expression of TRM markers and cytokines in CCR6⁺ CD127⁺ CD8⁺ TRM17 cells in HC or GVHD. Statistical difference in bar charts was calculated using unpaired t-test (p-value: * <0.05, *** <0.001), dots represent individual samples (n=4-6), samples with <20 cells in parent population were excluded from downstream gating.

Among CD4⁺ T cells, TRM17 identified by gating on CCR6⁺ CD127⁺ were also significantly reduced and displayed a decrease of GM-CSF and TNF- α but increased IFN- γ levels in GVHD

(Fig S3g-i). We also detected a TRM17 cluster by unsupervised analysis (CI 08), which was significantly enriched in healthy skin (Fig S3c-f). Although in single cell sequencing data *CCR6* and *IL7R* expression did not demarcate a transcriptionally separate TRM cluster as clearly as in CD8 skin T cells (Fig S2b,c), the TRM17 phenotype of CD4⁺ T cells still seemed to be disturbed in lesional skin. In summary, phenotypic and functional profiling of skin T cells confirmed the reduction of TRM17 in GVHD lesions, paralleled by an increase in CD49a and IFN- γ expressing TRM. This is consistent with our single-cell RNA sequencing data, showing a disbalance of TRM17 and TRM1 in type-1 dominated inflammation.

TRM17 recover after GVHD resolution

To evaluate the timeline of CD8⁺ TRM17 reduction, we leveraged circulating T cells with tissue-resident memory phenotype (cTRM, CD45RA⁻ CLA⁺ CD103⁺) as proxy for skin-resident T cells^{7,43} (Fig 6a,b). We first verified loss of CCR6⁺ CD127⁺ cells (cTRM17) among CD8⁺ cTRM in GVHD patients compared to healthy controls (Fig 6c), assuring that cTRM17 accurately reflect skin TRM17 dynamics. Next, we assessed CD8⁺ cTRM17 at baseline and D100 post-transplantation. As expected, we found a significant reduction in GVHD, but not at D100 (Fig 6d). Given that GVHD typically manifests between 14 and 100 days post-transplant, we further separated D100 samples into patients with a history of GVHD (GVHD dev), and patients without previous GVHD (GVHD free). While there was a trend towards fewer cTRM17 in GVHD dev patients, the difference was not statistically significant (Fig 6d), possibly due to small sample size, or rapid recovery of these cells after GVHD resolution.

To assess potential long-term effects in an additional cohort, we included 10 more patients which were at least one year after HSCT and either previously suffered from skin GVHD or were GVHD free. No significant differences were observed compared to healthy controls, and between the GVHD free and GVHD dev group among patients (Fig 6e). This suggests that CD8⁺ TRM17 depletion is acute, and that the population recovers within a few months after GVHD resolution.

Interestingly, while CD4⁺ TRM17 were depleted in lesional skin (Fig S3g), CD4⁺ cTRM17 in the blood did not show similar depletion (Fig S4a-c). This might reflect differences in the recirculation potential of CD8⁺ versus CD4⁺ TRM subsets.

To validate these findings with an additional method and assess their spatial distribution in the skin, we performed immunofluorescence analysis on skin sections from healthy controls, GVHD patients, and patients 2-9 years post-transplant (Fig 6f). Although CCR6 staining was unreliable due to technical limitations, we assessed CD127 expression on CD8⁺ skin T cells.

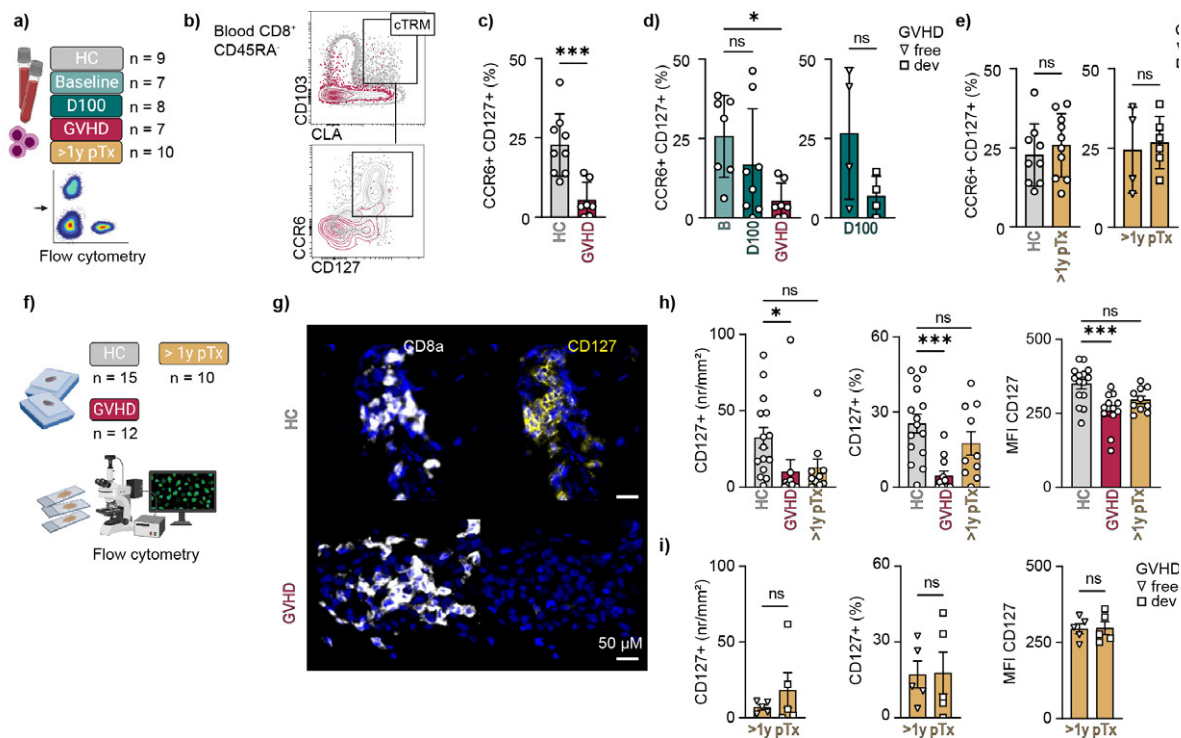


Figure 6 – Assessment of TRM17 depletion and recovery timeline before and after GVHD

a) Workflow scheme for profiling of blood cTRM in healthy controls (HC), HSCT patients at Baseline (B), D100 or 1-3 years after transplantation (>1y pTx), or GVHD patients. b) Representative gating plots to identify CCR6⁺ CD127⁺ cTRM17 among CD8⁺ CD45RA⁻ memory T cells in blood. c-e) Quantification of CCR6⁺ CD127⁺ cTRM17 cells among CD8⁺ cTRM in HC and GVHD (c), at Baseline, D100 and GVHD (d, left), GVHD free vs GVHD developers in D100 samples (d, right), in HC vs >1y pTx (e, left) and GVHD free vs GVHD developers in >1y pTx samples (e, right). f) Workflow scheme for expression analysis of CD127 and CD8a on dermal cells in fresh-frozen skin biopsies of HC, patients 2-9 years after transplantation (>1y pTx) or lesional skin of GVHD patients. g) Representative image of CD8a and CD127 staining. h) Quantification of density of CD8⁺ CD127⁺ cells (left), percentage of CD127⁺ among CD8⁺ cells (middle) and MFI of CD127 on CD8⁺ cells (right) in HC, GVHD and >1y pTx samples (n=10-15) i) Quantification of density of CD8⁺ CD127⁺ cells (left), percentage of CD127⁺ among CD8⁺ cells (middle) and MFI of CD127 on CD8⁺ cells (right) in GVHD free and GVHD developers within <1y pTx samples (n = 5). Statistical difference in bar charts was calculated using one-way ANOVA (comparison of 3 groups) or unpaired t-test (comparison of 2 groups), dots represent individual patients (p-value: * <0.05, *** <0.001).

Consistent with our previous findings, we observed a significant reduction in CD127⁺ CD8⁺ T cells and decreased CD127 expression in CD8⁺ T cells in the upper dermis of lesional GVHD skin, but no long-term reduction of TRM17 (Fig 6g-i).

Overall, these data show that while there is a specific and acute reduction and a functional impairment of TRM17 during ongoing GVHD inflammation, the population can be restored once inflammation is resolved and homeostasis is re-established.

DISCUSSION

Understanding T cell-mediated immune responses requires their study within tissues, where they occur. TRM play a role in all types of peripheral immune responses, including cancer, infection, vaccine responses, allergy and autoimmunity. In this study, we aimed to understand the developmental and functional heterogeneity of TRM populations in the skin, which is crucial for developing targeted strategies to selectively modulate these cells across diverse contexts.

We began by examining *de novo* generation of TRM from blood donor T cells after HSCT, aiming to identify which circulating cells give rise to newly formed skin TRM. In humans, the blood origins of TRM remain poorly understood. Mouse studies provide insights with lineage tracing experiments in skin vaccination models, indicating the existence of a TRM precursor population among circulating effector cells¹⁷ and systemic infection models identifying TRM precursors among early-stage effector cells in peripheral tissues^{20,44}. Both suggest TRM generation from the circulating effector T cell pool. On the other hand, in murine skin sensitization models, TRM show high clonal overlap with lymph node resident TCM, and studies with adoptive transfer in humanized mice indicate TCM as the most efficient skin TRM precursor^{18,45}. We could address this in a unique patient cohort after HSCT with single-cell transcriptomic and TCR profiling, enabling the tracing of newly formed TRM, based on their genetic origin. Our observation of clonal overlap almost exclusively between circulating donor TEM and newly established donor TRM strongly suggests that effector T cells give rise to TRM in human skin. Further studies are needed to determine if specific TRM subsets arise from distinct effector precursors.

Regarding TRM subset heterogeneity within the skin, we present strong evidence that CD8⁺ TRM17 are a developmentally distinct TRM population in human skin, which is selectively impaired in type 1-driven immune responses. While it is well established that early divergence to the type 17 lineage is repressed by type 1 transcription factors, such as T-bet or Eomes in IL-12, IFN- γ and type-I IFN rich environments^{26,40,46}, the development, regulation and turnover of type 17-polarized TRM in human skin are not well understood. Through clonal, genotypic and transcriptional analysis of established host- and newly formed donor-derived CD8⁺ TRM in human skin, we observed different maintenance and generation dynamics among distinct TRM populations following HSCT and in GVHD-related inflammation. Our data show that donor TRM readily form after transplantation but fail to replenish the TRM17 pool in patients with GVHD. Additionally, while certain host TRM may persist in human skin for up to 10 years⁸, host TRM17 appear less stable in the post transplantation period, particularly during type 1-dominated alloreactive inflammation.

This instability could be explained by different mechanisms. It is possible that host TRM17 cannot be maintained during exacerbated type 1 inflammation and are replaced by incoming donor TRM. In mice undergoing a secondary immune challenge newly generated antigen-specific CD8⁺ TRM preferentially replace existing bystander TRM, through competition for limiting amounts of active TGF- β ⁴⁷. In a similar manner, new donor T cells recruited to the skin could preferentially replace TRM17, which might be less fit to compete for survival factors in presence of strong type 1 stimuli. An alternative scenario is that due to signals in the inflamed skin, the identity of remaining host TRM17 is altered, resulting in a shift towards a dysfunctional, pathogenic type 1 phenotype with elevated IFN- γ expression. In naïve human CD4⁺ T cells, differentiation towards pathogenic IFN- γ ⁺ Th17 cells can be induced through uncontrolled type I IFN-signaling in dendritic cells, limiting active TGF- β ⁴⁸. We observed strong upregulation of type I IFN-related genes in DC in GVHD. Additionally, skin T cells significantly downregulated TGFBR3, encoding TGF- β receptor III, in GVHD. Interestingly, loss of TGF- β receptor III in murine CD4⁺ T cells increases inflammation in experimental autoimmune encephalomyelitis by promoting pathogenic IFN- γ ⁺ Th17 cells⁴⁹. Whether

signaling through these pathways may lead to reprogramming of differentiated TRM17 remains to be determined.

The exact signals driving these changes in the TRM repertoire during GVHD remain unknown but may involve a combination of cytokine- and receptor-mediated signaling from surrounding immune and non-immune cells, which suppress TRM17 generation and survival.

In mouse models, TRM17, generated alongside TRM1 in the same primary immune response to *Staphylococcus epidermidis*, decline fourfold within weeks, while TRM1 are stably maintained¹⁴. This decline is attributed to higher levels of Ki-67 and Bcl-2 in TRM1¹⁴. However, in these models, the abundance of *Staphylococcus epidermidis* diminishes over time, and although the authors demonstrated that the lack of antigen recognition via the TCR did not differentially affect the maintenance of TRM1 and TRM17¹⁴, the reduction in TRM17 may result from the absence of other TCR-independent survival signals.

Murine TRM17 are crucial regulators of commensal microbiota and generated after exposure to commensals^{50,51}. In humans, skin homeostasis involves constant microbial colonization, whereas in conditions like psoriasis lesions are characterized by microbial dysbiosis. In psoriasis, TRM17 are maintained long-term in resolved lesions, mediating recurrence of flares at the same skin sites⁶, and are even elevated in never lesional skin⁵. One could speculate that their maintenance in homeostatic conditions relies on tonic signaling in the tissue niche, induced by constant presence of commensal skin microbiota. This might be impaired in patients post transplantation due to the transient decrease of skin bacterial colonization after myeloablative conditioning⁵².

During GVHD, microbial dysbiosis, including *Staphylococcus spp* overgrowth⁵², raises the possibility that TRM17 reduction could lead to pathogenic microbial imbalances. After GVHD resolution, TRM17 numbers normalize to those seen in healthy human skin, indicating their rapid recovery in the absence of type 1-driven inflammation. Further research should clarify if TRM17 reduction contributes to GVHD pathology, and if replenishment of TRM17 would ameliorate disease, or if it is merely a side effect. Notably, we observed depletion of CD8⁺ cTRM17 also in blood as an easily accessible source for tissue sampling, suggesting time-course studies of patients after HSCT could reveal whether loss of CD8⁺ (c)TRM17 occurs

even before clinical GVHD manifestation. In this case cTRM17 could serve as a GVHD biomarker.

While CD4⁺ TRM17 in the skin were also decreased, their depletion was not mirrored in CD4⁺ cTRM in blood. This discrepancy could reflect different recirculation potentials for certain CD4⁺ and CD8⁺ TRM subsets in healthy and inflamed skin. In mice, studies have focused on CD8⁺ TRM retrograde migration upon secondary infection^{53,54}. However, CD4⁺ TRM have been described to recirculate both in homeostasis and inflammation in humans^{7,43}, though the pathways regulating retrograde migration in steady-state and inflammation, as well as in CD4⁺ versus CD8⁺ T cells remain unclear. We previously observed that skin inflammation increases host cells among cTRM, suggesting recirculation potential varies among TRM subsets⁷. During GVHD, non-lesional skin-derived cTRM may contribute more significantly to CD4⁺ TRM recirculating, potentially explaining the loss of TRM17 in CD8⁺ cTRM, while CD4⁺ cTRM17 remain stable.

Collectively, our study highlights the complexity of skin TRM biology in humans, and the dynamic changes this cell pool can undergo in inflammatory conditions. Our observation that CD8⁺ TRM17 are selectively depleted in human skin inflammation is intriguing, not only because it could be detrimental in GVHD, but also because these cells are essential in skin diseases, such as psoriasis. Although current treatments targeting cytokines, such as TNF- α , IL-17 or IL-23, are effective, directly depleting the cytokine-producing cells could offer greater therapeutic potential. Additionally, methods to tailor TRM functions might enhance cell therapies, such as CAR T cells for solid tumors⁵⁵. Ultimately, these insights will be instrumental to design precision therapy approaches in skin-related immune diseases and beyond.

MATERIALS AND METHODS

Human skin and blood samples

In this study, we collected blood and skin samples from patients receiving allogeneic hematopoietic stem cell transplantation and developing or not developing acute graft-versus-host disease. These patients were recruited at the Department of Medicine I and the

Department of Dermatology of the Medical University of Vienna (MedUni Vienna). Patients voluntarily participated with fully informed written consent, in line with national law and under an approved ethics vote (ECS 1087/2016). Samples from patients in the HSCT cohort were taken at different timepoints before and after transplantation (Baseline = 7 days prior to transplantation, before conditioning; Tx = day of transplantation, after conditioning; D14 = 14 days after transplantation; D100 = 91 – 101 days after transplantation); samples from HSCT patients in the GVHD cohort were taken at disease onset, before start of treatment and samples from HSCT patients in the >1y pTx cohort were taken at timepoints ranging from 1y – 9y after transplantation. Skin punch biopsies (6mm) were taken from healthy skin on the back or upper arms, or from lesional skin in the case of aGVHD. Patients detailed clinical information is listed in Table S1. Healthy control blood was collected from healthy volunteers. Healthy human skin was obtained from elective surgeries performed at the Department of Plastic and Reconstructive Surgery at MedUni Vienna under an approved ethics vote (ECS 1969/2021).

From whole blood, we isolated PBMCs on a Ficoll-Paque (Cytiva, GE17-1440-03) gradient. Cells were either cryopreserved in Fetal Bovine Serum (FBS) (Sigma-Aldrich, F4135) with 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, 472301) or used directly. Skin biopsies were split and one part fresh-frozen by embedding in OCT Compound Cryostat Embedding medium (Scigen, 23-730-625) and snap-freezing in liquid nitrogen for -80°C storage, and the other part processed directly for single-cell assays or cryopreserved in FBS with 10% DMSO.

Sample preparation for single-cell RNA and TCR sequencing

For droplet-based single-cell sequencing skin and blood samples were prepared according to our published protocol⁵⁶. Briefly, fresh or frozen skin was digested for 3 h at 37°C in a shaking water bath using the Whole Skin Dissociation Kit, human (Miltenyi Biotec, 130-101-540) with addition of Enzyme P. Skin and PBMC single-cell suspensions from individual patients were labelled for 30 min at 4°C with barcoded TotalSeq Hashtag antibodies and fluorescent antibodies against CD45 (see Table S2), and the viability dye 7-AAD (Invitrogen, A1310) to enable sorting of live CD45⁺ cells from skin and blood, as well as CD45⁻ cells from skin. Cells

were sorted at a BD FACS Aria into PCR tubes containing 25 µl of 0.08% Bovine Serum Albumin (BSA) (Sigma, A2153) in DPBS. After sorting skin and blood cells were pooled and processed on a 10X Genomics Chromium controller with 10X Genomics Chromium Next GEM Single Cell 5' Kit v2 (10x Genomics, 1000263), Chromium Next GEM Chip K Single Cell Kit (10X Genomics, 1000286), Chromium Single Cell Human TCR Amplification Kit (10X Genomics, 1000252) and 5' Feature Barcode Kit (10X Genomics, 1000256) according to the manufacturers protocol for construction of gene expression, TCR-αβ and feature barcode libraries. Gene expression and TCR libraries were indexed with Dual Index Kit TT Set A (10X Genomics, 1000250), feature barcode libraries with Dual Index Kit TN Set A (10X Genomics, 1000256). Pooled libraries were sequenced on an Illumina NovaSeq 6000 platform with 50bp paired-end dual index configuration at the Biomedical Sequencing Facility at CeMM, Vienna.

General single-cell RNA-sequencing data preprocessing

The single-cell RNA-sequencing data were processed by the BSF with Cell Ranger (v.7.1.0, 10x Genomics). Previously published single cell data from GVHD samples¹¹ were processed identical to the newly created data. The raw data were demultiplexed and converted to FASTQ format using *mkfastq* command. The sequencing reads were mapped per sample to the 10x Genomics human reference build GRCh38-2020-A with the *count* command. Demultiplexing of the tissue source feature barcodes (Blood or Skin, TotalSeq antibodies, see Table S2) was determined based on a Gaussian mixture model⁵⁷. The full Cell Ranger filtered count matrices were imported and merged using Seurat (v.4.3.0). The cells were pre-filtered by percentage mitochondrial reads ($\leq 15\%$) and unique molecular identifiers (UMIs) per cell (≥ 1000 counts) and stored as RDS or H5AD file for compatibility with R (v.4.1.0) and Python (v.3.8.19) respectively.

We used Scanpy⁵⁸ (v.1.9.6) for highly variable gene (HVG) selection, dimensional reduction, and Leiden clustering⁵⁹. Integration was carried out with scvi-tools⁶⁰ (v.0.20.3) single cell variational interference (scVI)⁶¹. All models were configured with a 10-dimensional latent space, two hidden layers with 128 nodes each, and a negative binomial gene likelihood distribution.

Doublet detection was carried out with the SOLO⁶² implementation from scvi-tools. The data were split by patients and subset by the top 2,000 HVGs with the Seurat method (v3)⁶³. For each subset a scVI model was trained for SOLO doublet prediction. A second scVI model was trained on the combined data. To cluster the cells, a nearest neighbor graph with 10 neighbors was calculated based on the latent space. Leiden clustering was done on the graph with a resolution of 1. For each cluster the doublet cell ratio was determined. To identify clusters that contained more doublets than expected we used the approach taken by Pijuan-Sala et al⁶⁴. For automated cell type annotation of individual cells, we used CellTypist⁶⁵ (v1.1.0) with majority vote disabled (*Immune_All_High.pkl* and *Immune_All_Low.pkl*).

The data were again filtered by the total number of UMIs per cell (≥ 1500 counts). To remove erythroid contamination, cells were filtered by percentage hemoglobin reads ($\leq 5\%$). In addition, cells associated with CellTypist erythroid cell type labels were filtered out. Cells labeled as doublet or without proper tissue demultiplexing label (Blood or Skin) were removed. The number of genes detected per cell was filtered per sample to be above the 1st percentile threshold. The count matrix was reduced to genes with at least three counts in one cell. At this step we recovered a total of 69,837 cells and 11,874 genes.

The data were now subjected to a first round of cluster cell type annotations to guide downstream preprocessing steps. For this purpose, a scVI model was informed about categorical (patient ID, time point, and digestion protocol) and continuous (percentage mitochondrial reads, difference between S-phase and G1-phase cell cycle score) covariates and trained for 200 epochs. To cluster the cells, a nearest neighbor graph with 30 neighbors was calculated based on the latent space. Leiden clustering was done on the graph with a resolution of 1. The Leiden clusters were annotated based on known markers and summarized as domain labels (lymphocyte, myeloid, and non-immune cells).

Host/Donor genotype demultiplexing

The read alignment BAM files were subset by the barcodes that passed the 10x Cell Ranger quality thresholds and merged per patient using mergeBams (v.0.14). SNPs were detected with cellsnp-lite⁶⁶ (v.1.2.2) and the genome1K reference variant call format (VCF:

genome1K.phase3.SNP_AF5e4.chr1toX.hg38.vcf.gz). The read coverage threshold was set to 20 and no SNPs were removed from the reference VCF based on allele frequency. The result files were converted into vartrix format and filtered by minor allele frequency ($MAF \geq 0.01$) using custom R scripts. For cell genotype demultiplexing we utilized vireoSNP¹⁹ (v.0.5.7) with N set to two and 100 random initialization. To annotate generic vireoSNP genotypes (0, 1) with Host/Donor labels we utilized ground truth information of the data structure. Cells from timepoints Baseline and Tx, as well as non-immune cells served as control for the Host genotype. The vireoSNP genotype that was enriched within this control subset was set to Host and the remainder to Donor. Cells marked as duplicate or failed genotype prediction were removed from the dataset. To determine variants with the highest predictive power for genotype demultiplexing we utilized GTbarcode from vireoSNP.

High resolution Leiden cluster annotation

The first task was to detect remaining low-quality cells or cell states that are not shared between patients. To address this, we implemented a Scanorama⁶⁷ (v.1.7.3) workflow that has shown to conserve high amounts of biological signal⁶⁸. To reduce complexity, the cells were split by the previously computed domain labels (lymphocyte, myeloid, non-immune) and processed separately. Genes with fewer than three counts in three cells were omitted. Data normalization was carried out with scran⁶⁷ (v.1.22.1). Since the scran pooling method relies on clusters, we used 15 principal components computed on the $\log(TPM+1)$ normalized counts for neighbor graph computation and Leiden clustering with a resolution of 0.5. The cluster vector was then used with the scran computeSumFactors to derive size factors. The raw counts were then divided by the size factor and log normalized. HVGs were selected using Scanpy with the Cell Ranger⁶⁹ method with minimal dispersion of 0.5 and the batch key set to sample. The HVGs from samples with more than 150 cells were then combined into a consensus HVG set. Subsequently the scran normalized counts were scaled, subset by the HVGs, and split by sample. The AnnData list was then integrated with the Scanorama function integrate_scanpy on 100 principal components. The results were combined to obtain the integrated Scanorama embedding which was used as input for nearest neighbor graph

construction with 15 neighbors. Leiden clustering was performed on the graph with a resolution of 2.

Across all data sets we observed small clusters with high percentage of mitochondrial reads, low percentage of ribosomal reads, and low numbers of detected genes per cell. The clusters potentially represent damaged cells or single nuclei. The respective cluster and similar cells with high percentage mitochondrial reads ($> 10\%$) together with low percentage of ribosomal reads ($\leq 7.5\%$) were removed. Small clusters that were dominated by a single patient were also removed upon visual inspection.

The second task was to generate the actual high-resolution embedding. The lymphocyte and myeloid domain cells were further split by tissue origin (Blood and Skin) and the count matrix filtered to retain only genes with a minimum of three counts in at least three cells. A scVI model was trained for each of the five resulting groups. The model was informed about the batch (sample name) and continuous covariates (percentage mitochondrial reads, difference between S-phase and G1-phase cell cycle score). Leiden clusters were derived from the latent space as described previously with 30 neighbors for graph construction and cluster resolution of 2. Each cluster was then manually annotated based on marker gene expression and supported by the CellTypist predictions. The annotated cell type plus Leiden cluster number is stored in the `leiden_cell_type_fine` column of the meta data. Choosing a high-resolution clustering approach enables us to detect rare cell types and states. However, that approach is prone to splitting the same cell type into multiple clusters. Hence, when we had no evidence that a cluster represents a distinct state we merged the respective cluster into a main annotation. The result is stored in the `leiden_cell_type_main` column of the meta data. In total we detected 35 main cell types in 60,019 cells that were used for all further downstream analysis. The uniform manifold approximation and projection (UMAP) presented Figure 1 is derived from scVI models of the combined data. UMAPs in Figure 2 and Figure S1 are derived from scVI models of all lymphoid, myeloid or non-hematopoietic cells respectively. They were processed as previously described and the UMAP was calculated on the neighbor graph based on the latent space.

To detect TRM17 clusters, we split cells into T cells CD4⁺ (Th) and T cell CD8⁺ (Tc) subsets based on the high-resolution annotation. In addition, cycling cells that were labeled by CellTypist as helper T cells or cytotoxic T cells were added to the subset respectively. We trained a scVI model as described before and derived a graph with 15 neighbors from the latent space for Leiden clustering at resolution 2. The partition-based graph abstraction²⁸ (PAGA) was computed on the Leiden cluster with Scanpy model v1.2, disabled thresholding of the connections, and the Fruchterman-Reingold layout. The respective UMAPs were initialized on the PAGA position to match the embedding. The MS of *CCR6/IL7R* expression was scored with Scanpy and min-max normalized.

TCR analysis

For TCR analysis we utilized Scirpy⁷⁰ (v.0.12.0). The αβ-TCR raw data were imported from the Cell Ranger output with the read_10x_vdj function and combined across all samples. Cells with annotated single pair, extra VJ, extra VDJ, and two full chains were used for downstream analysis. A distance matrix based on the pairwise amino acid sequence alignments was calculated with a distance cut-off of 10. Based on the results clonotyping was carried out using default parameters. The clone id (cc_aa_alignment) and alignment size (cc_aa_alignment_size) was attached to the meta data and used for the analysis presented in this manuscript.

Differential expression analysis (DEA)

For DEA we employ the edgeR⁷¹ (v.3.36.0) function voomLmFit to moderate abundant zero counts and to enable Limma⁷² (v.3.50.3) precision weights with blocking of the patient ID as random effect. For the time point analysis, we used pseudobulks and for all other comparisons pseudoreplication as input data^{73,74}. Briefly, the data was first split by cell type. For each covariant we tested representation of at least three patients. If not, the respective group of the covariant was removed and no comparison was conducted. The count matrix was then filtered to only retain genes with three counts in at least three patients. If applicable, the gene counts were now summed into pseudobulks for each patient. For each cell type a linear model was constructed with the voomLmFit function with the design ~0+group and the block parameter

set to the patient ID vector. The comparisons of interest were formulated as contrast vector and the result statistics were computed with the Limma eBayes function. All filter options of the topTable function were disabled to generate the result table. LogFC with adjusted p-value ≤ 0.05 were defined as significant.

Gene set enrichment analysis

Gene set enrichment was performed on significant differentially expressed genes using EnrichR⁷⁵, extracting MSigDB Hallmark 2020 pathways, with p-value < 0.05.

Gene-regulatory network analysis (GRN)

For the GRN analysis we used pyscenic^{76,77} (v.0.12.1) with the GRNBoos2⁷⁸ algorithm and followed the Nature Protocol⁷⁷. The analysis was performed only on the lymphoid domain subset.

Phenotyping of skin and blood cells by flow cytometry

For phenotyping of surface marker expression of T cells from blood, cryo-preserved PBMCs were thawed and immediately stained for flow cytometry. For cytokine expression assays, frozen or fresh skin biopsies were digested as described above using the Whole Human Skin Digestion Kit (Miltenyi Biotec, 130-101-540). Single cell suspensions were resuspended in 200 μ l RPMI1640 Medium (Gibco, 52400-025) with 1% Penicillin/Streptomycin (Gibco, 15140122) and 10%, plated in a 96-well flat bottom plate and rested over night at 37°C 5% CO₂. Then cell suspensions were treated with 1X Cell Activation Cocktail with Brefeldin A (Biolegend, 423303) and incubated for 4h at 37°C 5% CO₂. For flow cytometric staining, cells were incubated with surface receptor antibodies in DPBS (Gibco, 14190-169) for 20 min on ice and fixed for 20 min at room temperature with Fixation Buffer (Biolegend, 423304). For intracellular cytokine staining, cells were permeabilized by 15 min incubation and subsequent washing in 1X Intracellular Staining Permeabilization Wash Buffer (Biolegend, 421002) and then stained with cytokine-targeting antibodies in the same buffer for 40 min on room temperature. For transcription factor staining, after surface receptor staining, cells were stained fixed and permeabilized with the eBioscience Foxp3/Transcription Factor Staining Buffer Set (Invitrogen, 00-5523-00) according to manufacturer's protocol, by staining with transcription factor-

targeting antibodies for 1 h at room temperature. Stained samples were acquired on a CYTEK Aurora Spectral flow cytometer (5 Laser) at the Flow Cytometry Core Facility of MedUni Vienna. Data was analyzed using FlowJo (v10.9.0) or OMIQ (Dotmatics) software. Conventional gating was done in FlowJo or OMIQ using fluorescence-minus-one samples and unstimulated samples as gating controls. Calculations of opt-SNE embedding and Phenograph clusters were run on FDA-normalized data in OMIQ with software-integrated packages. EdgeR package in OMIQ was used to determine cluster enrichment. Antibodies used in these experiments are listed in Table S2.

Immunofluorescence profiling of human skin

For *in situ* staining of skin T cells we prepared skin sections of OCT-embedded fresh-frozen tissue. Sample blocks were cut to 6-8 μM sections on a Leica Cryostat and acetone-fixed for cryo-preservation. For staining, slides were blocked with 2% goat serum (Dako, X0907) and stained with primary unlabeled or fluorescently labelled antibodies, or isotype-matched control antibodies for 2h at room temperature or overnight at 4°C and subsequently stained with appropriate secondary antibodies for 30 min at room temperature in DPBS with 2% BSA. Antibodies used in these experiments are listed in Table S2. Slides were counterstained for 5 min with DAPI (Sigma, D9542-1MG) and mounted with PermaFluor™ Epredia™ Lab Vision™ aqueous mounting medium (ePredia, TA-030-FM). Slides were stored at 4°C and acquired within seven days on a TissueFAX inverted fluorescent slide scanner microscope (TissueGnostics) with the 40X objective and analyzed using TissueQuest 7 software (TissueGnostics). For TissueQuest analysis, in every section a region of interest spanning the dermis ~500 μM deep from the epidermal basal layer was manually defined for analysis, excluding vessel structures and hair follicles. From every patient at least 4 sections or a minimum of 1 mm^2 was analyzed.

Statistical analysis of immunofluorescence and flow cytometry data

Output data from flow cytometry and immunofluorescence analysis software was imported in GraphPad Prism 9.0 software, which was used for plotting and statistical analysis with software-integrated appropriate statistical tests, indicated in the respective figure legends.

List of Supplementary Materials

Table S1 – Extended clinical information of included patients

Table S2 – Antibodies uses for flow cytometry and immunofluorescence

Figure S1 – Sub-clustering and annotation of myeloid and non-hematopoietic cells

Figure S2 – CD8 and CD4 T cell sub-clustering and transcriptional analysis

Figure S3 – Phenotypic and functional profiling of TRM17 in healthy and lesional skin

Figure S4 – CD4⁺ cTRM17 analysis

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Author contributions: GS, LMG and FD conceptualized the study. GS supervised the study and provided funding. LT and WR coordinated patient recruitment. LT, JS, AG, LK, CF, CM, LZR and JC carried out sampling of patients and healthy controls. FD performed bioinformatics analysis. RDH, LES and MF processed samples for single-cell sequencing. BR and LK performed experiments. LMG performed sample processing, bioinformatics analysis, experiments and formal analysis. LMG prepared figures. LMG, GS and FD wrote the manuscript. All authors critically reviewed and approved the final version of the manuscript.

Competing interest: The authors declare no competing interest.

Data and materials availability: All count matrices of single-cell RNA and TCR sequencing data generated in this study will be made available upon publication in the NCBI Gene Expression Omnibus database (GEO) under accession number GSE280571, including reprocessed files (Cell Ranger v7.1.0) from our previously published dataset¹⁵ (GSE236264, Cell Ranger v3.0.2). Raw single cell RNA and TCR sequencing data will be uploaded to the European

Genome-Phenome Archive (EGA). Codes used for analyses performed in this paper, as well as the integrated anndata object corresponding to the UMAPs in Figure 1 will be made available on Zenodo.

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Tables

Table S1 – Extended clinical information of included patients

Clinical data of all patients included in this study. Abbreviations of allogeneic hematopoietic stem cell transplant (HSCT) indication: AML = Acute myeloid leukemia, ALL = Acute lymphoblastic leukemia, DLBCL = Diffuse large B cell lymphoma, CML = Chronic myeloid leukemia, MDS = myelodysplastic syndrome, MPN = myeloproliferative neoplasm. Patients received reduced-intensity or myeloablative conditioning according to standard clinical protocols, usually a combination of treatments including Busulfan (B, Bu) Fludarabine (F, Flu), Thiothepa (T), Treosulfan (Treo), Cyclophosphamide (Cy) or total body irradiation (TBI). Drugs used for Graft-versus-host Disease (GVHD) prophylaxis included Cyclosporin A (CSA), Mycophenolate Mofetil (MMF), post-transplant Cy (PTCy). Other abbreviations: aGVHD = acute GVHD, cGVHD = chronic GVHD, GI GVHD = gastrointestinal GVHD, f = female, m = male, HLA = human leukocyte antigen, URD = unrelated donor.

ID	Sex	Age at sampling	Group	HSCT indication	Conditioning	Donor	Sex mismatch	Skin aGVHD	Skin aGVHD onset	GVHD prophylaxis	Other GVHD representation until sampling	Analysis for paper
P1	m	55	HSCT	AML	myeloablative, TBSF	haploident sibling	yes	no	na	PTCy, CSA/MMF	no	scRNAseq
P2	m	63	HSCT	MDS	myeloablative, FB3	HLA-ident sibling	yes	no	na	CSA/MMF	no	scRNAseq
P3	f	27	HSCT	AML	myeloablative, FB3	HLA-matched URD	yes	no	na	CSA/MMF	GI aGVHD, d65	scRNAseq, cTRM profiling
P4	f	56	HSCT	AML	myeloablative, FB3	HLA-matched URD	yes	no	na	CSA/MMF	no	scRNAseq, cTRM profiling
P5	m	40	HSCT	AML	myeloablative, FludB4	HLA-ident sibling	yes	no	na	CSA/MMF/ATG	no	scRNAseq, cTRM profiling
P6	m	62	HSCT, GVHD	AML	myeloablative, TBSF	haploident daughter	yes	yes	d20	PTCy, CSA/MMF	no	scRNAseq, cTRM profiling
P7	f	31	HSCT	AML	myeloablative, TBSF	HLA-matched URD	yes	yes	d61 (not sampled)	PTCy, CSA/MMF	no	scRNAseq, cTRM profiling
P8	m	66	GVHD	MPN	myeloablative, Flut/Treo30	HLA-matched URD	no	yes	d60	PTCy, Balmore	no	scRNAseq, cTRM profiling
P9	m	57	GVHD	MM	myeloablative, Flut/Treo30	HLA-matched URD	no	yes	d60	CSA/MMF/ATG	no	scRNAseq
P10	m	59	GVHD	MDS	myeloablative, Flut/Treo30	HLA-matched URD	no	yes	d46	CSA/MMF/ATG	no	scRNAseq
P11	f	55	GVHD	MDS	myeloablative, other	HLA-ident sibling	yes	yes	d160 (alc overlap)	PTCy, Balmore	Oral aGVHD	skin TRM profiling, cTRM profiling
P12	f	30	GVHD	ALL	myeloablative, Flut/TB8-12	HLA-matched URD	no	yes	d26	CSA/MMF/ATG-F	no	skin TRM profiling
P13	f	58	GVHD	MDS	myeloablative, Flut/Treo30	HLA-ident sibling	no	yes	d154 (alc overlap)	CSA/MMF/ATG	no	skin TRM profiling
P14	f	45	GVHD	AML	myeloablative, Flut/Treo30	HLA-matched URD	no	yes	d25	CSA/MMF/ATG	no	skin TRM profiling
P15	f	57	GVHD	MDS	myeloablative, Flut/Treo30	HLA-ident sibling	no	yes	d61	CSA/MMF/ATG	no	skin TRM profiling
P16	m	55	HSCT	AML	myeloablative, TBSF	haploident sibling	yes	yes	na	CSA/MMF/ATG-F	no	skin TRM profiling
P17	f	61	HSCT	AML	myeloablative, TBSF	HLA-matched URD	yes	yes	d83 (not sampled)	CSA/MMF/ATG-F	no	skin TRM profiling
P18	m	76	HSCT	AML	myeloablative, Flut/Treo30	HLA-matched URD	yes	yes	na	CSA/MMF/ATG-F	no	skin TRM profiling
P19	f	57	GVHD	MDS	myeloablative, FludB4	HLA-matched URD	yes	yes	d63	CSA/MMF/ATG-F	no	skin TRM profiling
P20	m	69	GVHD	AML	myeloablative, TBSF	haploident son	no	yes	d42	PTCy, Balmore, CSA/MMF	no	skin TRM profiling
P21	f	43	GVHD	AML	myeloablative, FludB4	HLA-matched URD	no	yes	d80	PTCy, Balmore, CSA/MMF	no	skin TRM profiling
P22	m	51	GVHD	DLBCL	myeloablative, Flut/TB8-12	HLA-ident sibling	yes	yes	d63	CSA/MMF/ATG-F	no	skin TRM profiling
P23	m	67	GVHD	Myelofibrosis	myeloablative, FB3	HLA-matched URD	no	yes	d14	CSA/MMF	no	IF, cTRM profiling
P24	f	53	GVHD	MM	myeloablative, Flut/Treo30	HLA-matched URD	no	yes	d42	CSA/MMF/ATG-F	no	IF
P25	m	56	GVHD	Non-Hodgkin Lymphoma	reduced intensity, other	HLA-matched blood cord	no	yes	d21	CSA/MMF	no	IF
P26	m	50	GVHD	DLBCL	reduced intensity, other	HLA-matched URD	no	yes	d20	CSA/MMF	liver aGVHD	IF
P27	m	41	GVHD	AML	reduced intensity, other	HLA-matched URD	yes	yes	d22	CSA/MMF	eye liver GI aGVHD	IF
P28	f	20	GVHD	c-ALL	myeloablative, Flut/Treo30	HLA-matched URD	yes	yes	d60	CSA/MMF	no	IF
P29	f	56	GVHD	B-ALL	myeloablative, Flut/TB8-12	HLA-matched URD	yes	yes	d29	CSA/MMF/ATG-F	Gliver aGVHD	IF
P30	m	59	GVHD	AML	myeloablative, Flut/Treo30	HLA-matched URD	yes	yes	d35	CSA/MMF/ATG-F	GI aGVHD	IF
P31	m	49	GVHD	AML	myeloablative, TBSF	HLA-matched URD	yes	yes	d63	PTCy, Balmore, CSA + MMF	no	IF
P32	f	53	> 1y pTx	AML	myeloablative, Flut/Treo30	HLA-matched URD	yes	no	na	CSA/MMF/ATG-F	Gliver aGVHD	cTRM profiling
P33	m	55	> 1y pTx	AML (BCR-ABL)	myeloablative, Cy/TBI (13.2Gy)	HLA-matched URD	no	no	na	CSA/MMF/ATG-F	no	cTRM profiling
P34	m	67	> 1y pTx	AML (BCR-ABL)	myeloablative, FludB4	HLA-matched sibling	yes	no	na	CSA/MMF/ATG-F	Eye/liver cGVHD	cTRM profiling
P35	f	47	> 1y pTx	AML	myeloablative, other	HLA-matched URD	yes	no	na	CSA/MMF	no	cTRM profiling
P36	m	34	> 1y pTx	ALL	myeloablative, Flut/TB8-12	HLA-mismatched URD	yes	yes	d39	PTCy, CSA/MMF	no	cTRM profiling
P37	m	49	> 1y pTx	Myeloid sarcoma	myeloablative, Flut/Treo30	HLA-ident sibling	yes	yes	d32	CSA/MMF/ATG-F	no	cTRM profiling
P38	f	40	> 1y pTx	AML	myeloablative, FludB4	HLA-matched URD	yes	yes	d37	CSA/MMF/ATG-F	no	cTRM profiling
P39	f	56	> 1y pTx	AML	reduced intensity, other	HLA-matched URD	yes	yes	d31	CSA/MMF/ATG-F	no	cTRM profiling
P40	f	57	GVHD, > 1y pTx	AML	myeloablative, FB3	HLA-ident sibling	yes	yes	d16	CSA/MMF/ATG-F	no	cTRM profiling
P41	m	59	> 1y pTx	MDS	myeloablative, FB3	HLA-matched sibling	no	yes	d17	CSA/MMF/ATG-F	no	cTRM profiling
P42	m	56	> 1y pTx	Myelofibrosis	myeloablative, FB3	HLA-mismatched URD	yes	no	na	CSA/MMF/ATG-F	no	cTRM profiling
P43	m	30	> 1y pTx	pro-B-ALL	myeloablative, Cy/TBI (13.2Gy)	HLA-matched URD	yes	no	na	CSA/MMF	no	IF
P44	m	41	> 1y pTx	AML	myeloablative, Cy/TBI (13.2Gy)	HLA-mismatched URD	yes	no	na	CSA/MMF	no	IF
P45	f	44	> 1y pTx	AML	myeloablative, Cy/TBI (13.2Gy)	HLA-matched URD	yes	no	na	CSA/MMF	GI GVHD	IF
P46	f	45	> 1y pTx	MDS	myeloablative, Cy/TBI (13.2Gy)	HLA-matched URD	yes	no	na	CSA/MMF	no	IF
P47	f	49	> 1y pTx	AML	myeloablative, Cy/TBI (13.2Gy)	HLA-matched URD	yes	yes	d8	CSA/MMF	GI aGVHD, eye cGVHD	IF
P48	f	49	> 1y pTx	AML	myeloablative, Cy/TBI (13.2Gy)	HLA-matched URD	yes	yes	d13	CSA/MMF	Eye cGVHD	IF
P49	f	49	> 1y pTx	AML (BCR-ABL)	myeloablative, Cy/TBI (13.2Gy)	HLA-matched URD	yes	yes	d8	CSA/MMF	Eye aGVHD	IF
P50	m	47	> 1y pTx	ALL	myeloablative, Cy/TBI (13.2Gy)	HLA-matched URD	yes	yes	d12	CSA/MMF	Eye cGVHD	IF
P51	m	26	> 1y pTx	AML (BCR-ABL)	myeloablative, Cy/TBI (13.2Gy)	HLA-mismatched URD	yes	yes	d20	CSA/MMF	Liver cGVHD	IF

Table S2 – Antibodies uses for flow cytometry and immunofluorescence

Antibodies and dyes used in this study for sorting and demultiplexing skin cells and PBMCs for single-cell RNA sequencing, phenotypic profiling of skin cells and PBMCs by flow cytometry, and immunofluorescence staining on cryopreserved skin sections.

Antibody	Company	Catalogue number	Dilution
anti-human CCR6 BV785	Biolegend	353422	1:100
anti-human CD103 BUV805	BD	748501	1:400
anti-human CD127 Alexa Fluor 700	Biolegend	351344	1:500
anti-human CD127	Invitrogen	14-1278-82	1:1000
anti-human CD25 APC	Miltenyi Biotec	130-113-846	1:25
anti-human CD3 PerCp	Biolegend	344814	1:300
anti-human CD4 BUV563	BD	750979	1:200
anti-human CD45 BV57	Biolegend	304034	1:100
anti-human CD45 PE-CF594	BD	563379	1:200
anti-human CD45RA BUV395	BD	740315	1:400
anti-human CD49a APC/Fire750	Biolegend	328318	1:500
anti-human CD69 BUV395	BD	654364	1:100
anti-human CD8a Pacific Blue	Biolegend	344718	1:100
anti-human CD8a BV421	Biolegend	301036	1:100
anti-human CD8a Alexa Fluor 750	R&D	FAB1509RS-100UG	1:20
anti-human CXCR6 PE-Cy7	Biolegend	356011	1:200
anti-human GM-CSF PE/Dazzle594	Biolegend	502318	1:50
anti-human ICOS BV480	BD	746248	1:300
anti-human IFN-gamma FITC	Biolegend	502506	1:500
anti-human IL-17A BV421	Biolegend	512322	1:25
anti-human IL-22 PE	R&D	142928	1:50
anti-human TNF-alpha BV605	Biolegend	502936	1:100
anti-mouse IgG1 Alexa Fluor 647	Invitrogen	A21240	1:1000
mouse IgG1,k Isotype control	BD	349040	1:100
mouse IgG2b Alexa Fluor 750 Isotype control	R&D	IC0041S	1:100
TotalSeq™-C0251 anti-human Hashtag 1 Antibody	Biolegend	394661	1:25-100
TotalSeq™-C0252 anti-human Hashtag 2 Antibody	Biolegend	394663	1:25-100
TotalSeq™-C0253 anti-human Hashtag 3 Antibody	Biolegend	394665	1:25-100
TotalSeq™-C0254 anti-human Hashtag 4 Antibody	Biolegend	394667	1:25-100

Supplementary Figures

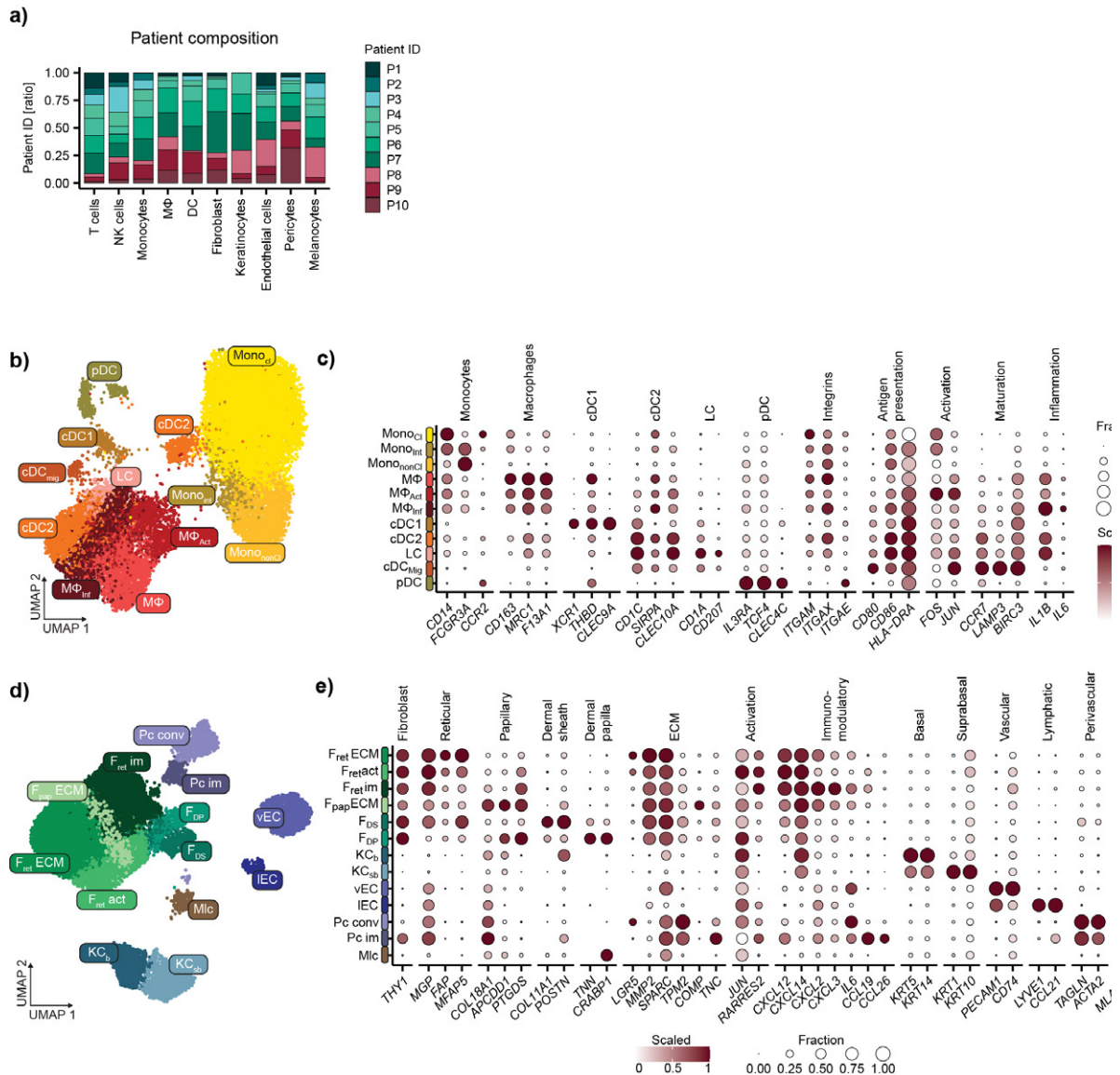


Figure S1 – Sub-clustering and annotation of myeloid and non-hematopoietic cells

a) Bar chart of patient sample origin per cell group in single-cell RNA sequencing data. b-e) UMAPs of myeloid cells (b) and non-hematopoietic cells (d) after scVI integration and Leiden clustering, cluster labels identify cell states annotated by expression of marker genes shown in dot plots (c – myeloid cells, e – non-hematopoietic cells).

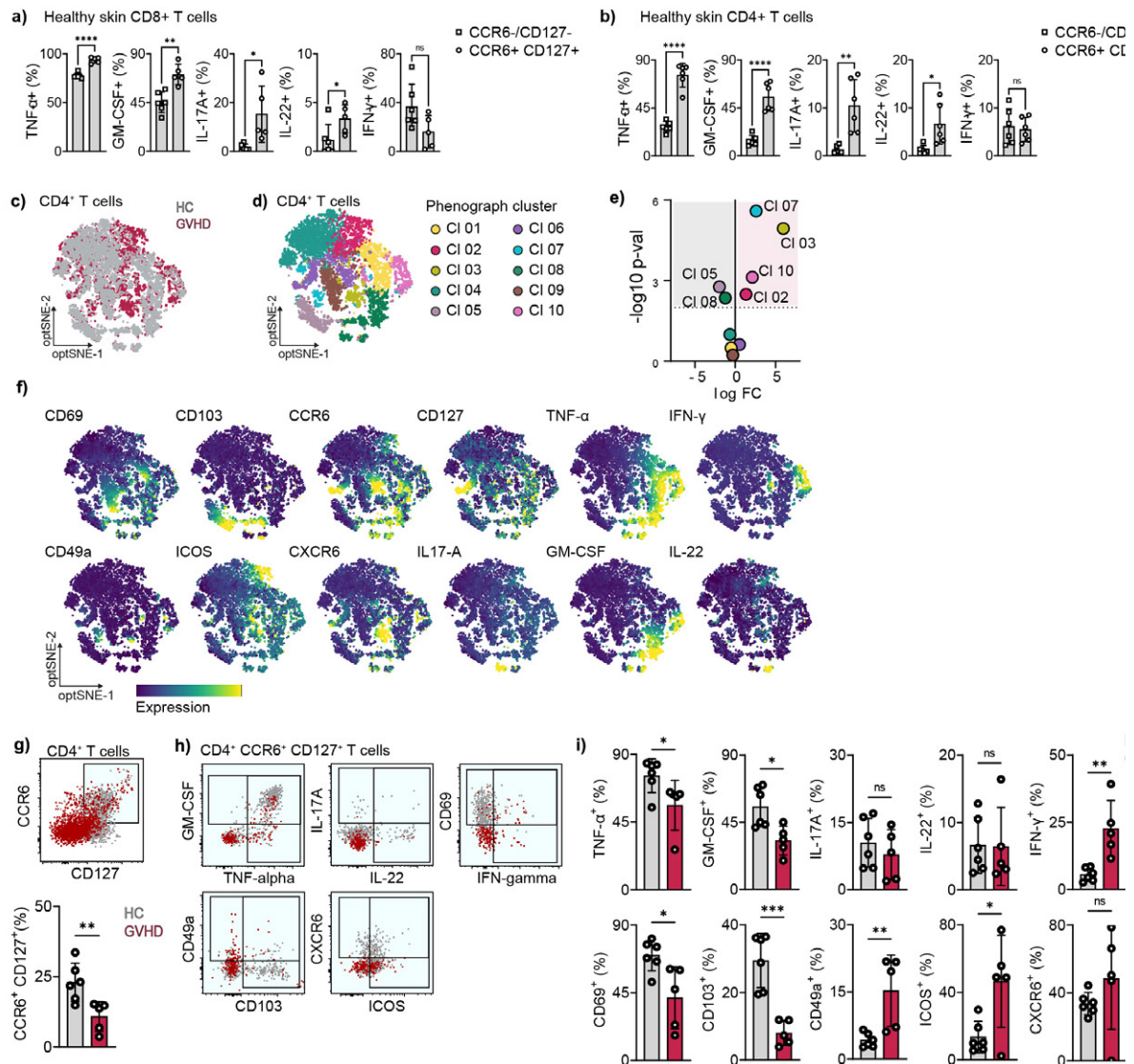


Figure S3 – Phenotypic and functional profiling of TRM17 in healthy and lesional skin

Quantification of cytokine expression comparing CCR6⁺ CD127⁺ TRM17 cells and CCR6⁻/CD127⁻ T cells among skin CD8⁺ (a) and skin CD4⁺ (b) T cells in healthy human skin. c-d) Opt-SNE plot of all skin CD4⁺ T cells colored by sample group (c) or Phenograph cluster ID (d). e) Volcano plot of differentially abundant clusters in HC or GVHD skin, calculated with EdgeR, p-value cutoff < 0.01. f) Opt-SNE plot of all skin CD4⁺ T cells colored by scaled expression of assayed proteins used for calculation of opt-SNE and phenograph clusters. g) Representative gating plot and quantification of CCR6⁺ CD127⁺ TRM17 cells among CD4⁺ T cells in HC and GVHD. h-i) Representative gating plots (h) and quantification (i) of expression of TRM markers and cytokines in CCR6⁺ CD127⁺ CD4⁺ TRM17 cells in HC or GVHD. Statistical difference in bar charts was calculated using unpaired t-test (p-value: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001), dots represent individual samples (n=4-6), samples with <20 cells in parent population were excluded from downstream gating.

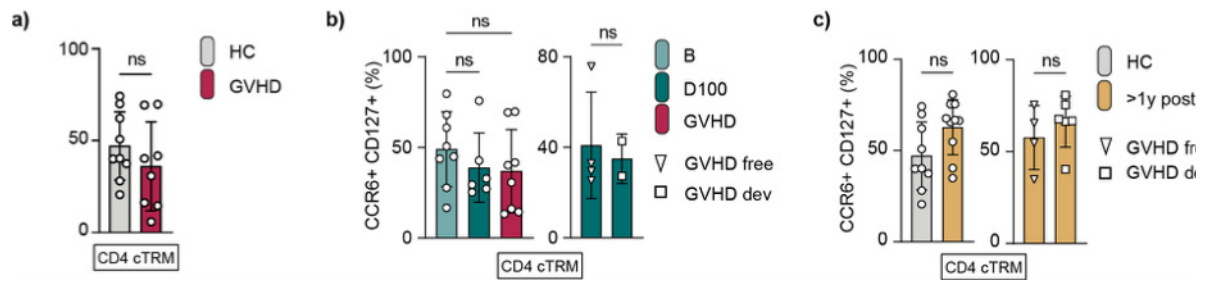


Figure S4 – CD4⁺ cTRM17 analysis

Quantification of CCR6⁺ CD127⁺ cTRM17 cells among CD4⁺ cTRM in HC and GVHD (a), at Baseline, D100 and GVHD (b, left), in GVHD free vs GVHD developers in D100 samples (b, right), in HC vs >1y pTx (c, left) and GVHD free vs GVHD developers in >1y pTx samples (c, right). Statistical difference in bar charts was calculated using one-way ANOVA (comparison of 3 groups) or unpaired t-test (comparison of two groups), dots represent individual patients, p-value cutoff < 0.05.

3. Discussion

3.1. General discussion

TRM have emerged as the central regulators of T cell-mediated immune responses in peripheral tissues, highlighting the limitations of relying solely on blood-derived T cell studies to fully comprehend tissue-specific immune responses. In this thesis, I aimed to get a better understanding of human TRM biology in the skin. This work builds on a previous study from our lab, demonstrating that host TRM survive radio-chemotherapy and are associated with the development of aGVHD after HSCT¹³⁹. Here, we studied TRM in the skin of these patients to address questions of TRM development, maintenance and migration in the absence of ongoing skin inflammation after HSCT, as well as their functional properties in aGVHD as classical T cell-mediated inflammatory skin disease.

Novel perspectives on TRM biology and skin immunity

The work presented in this thesis uncovered important aspects of TRM biology and skin immunity. Our key findings include the high clonal overlap between donor TEM and newly formed skin TRM, strongly suggesting that *de novo* TRM are developing from TEM and not TCM, helping to resolve an ongoing debate in the field. Moreover, we demonstrated the existence of a small, partially host TRM-derived subset of recirculating T cells (cTRM) after HSCT. Host cTRM are elevated in aGVHD, can cause damage to non-hematopoietic skin cells *ex vivo*, and have homing properties that enable migration to distant tissue sites. This is challenging the concept of inside-out donor-versus-host directed immune responses being the sole mediators of pathology in aGVHD. Using high-dimensional analysis tools for single-cell transcriptomics, we further demonstrated that heterogeneous skin TRM populations are likely developing on independent transcriptional trajectories, as we observed a strong bias towards TRM1 development and a severe reduction of TRM17 development and maintenance in aGVHD.

In addition to these discoveries on human TRM biology, we also generated the first single-cell transcriptomics dataset of its kind, integrating time-, tissue-, genotype- and clonotype-resolved cellular profiles from 10 patients and 24 samples. In our study we focused on T cells, but the data includes myeloid and non-hematopoietic cells and will be publicly available upon publication of the second manuscript. This dataset will thus be a valuable resource for researchers working on HSCT and aGVHD, or inflammatory skin diseases and tissue immunology in general. We also provide a detailed framework for in-depth single-cell RNA sequencing analysis, including high-confidence genotype annotation based on SNPs, which could be useful to many researchers working in the field of transplantation.

While our studies provide valuable insights into human skin immunity, they also raise several new questions and highlight contradictions that still need to be resolved in future studies. The individual manuscripts include brief discussion sections relating to their specific findings. Here,

I want to put our key observations in a broader context and explain the remaining knowledge gaps and emerging concepts brought forward by our results. I will summarize inconsistencies and questions regarding TRM undergoing retrograde migration in mice (ex-TRM) and humans (cTRM) and concerning phenotypic and functional heterogeneity of TRM1 and TRM17 observed in different studies. I will also contextualize the relevance of our findings for improving pre-clinical research on HSCT and aGVHD development and advancing precision medicine approaches in general. Lastly, I want to give an outlook on the future of immunological research in human tissues.

Understanding mechanisms of retrograde migration to infer tissue TRM states from blood

While skin is a relatively easily accessible organ, there are limitations to collecting large tissue pieces or repeated biopsies from patients, and for internal organs, for obvious reasons, it is much harder to obtain primary tissue material. As we also demonstrated here, human TRM can undergo retrograde migration and likely mediate outside-in immune responses. These specific cells could represent an exciting opportunity to study tissue immunity by-proxy in the blood. Human cTRM (CD45RA⁻ CLA⁺ CD103⁺ blood T cells) have been previously shown to represent progeny of bona fide skin TRM in humans⁹¹, and likely represent a subset of the ex-TRM population described in mice.

In this thesis, we wanted to explore retrograde migration behavior of skin TRM in the context of HSCT and aGVHD. For this purpose, we performed longitudinal analysis of cTRM in patients following HSCT and aGVHD. Our study (2.1.1.) demonstrated that cTRM can mirror host/donor TRM chimerism dynamics after HSCT, while in the remaining blood T cells host cells were virtually absent. In aGVHD, cTRM expressed similar cytokines as their skin TRM counterparts. Moreover, during skin inflammation the proportion of host cells among cTRM was significantly increased, suggesting a distinct activation of retrograde migration in host TRM in lesional skin. In our second study (2.2.1.), we observed that CD8⁺ cTRM17 mirrored the reduction of CD8⁺ TRM17. However, this was not the case for TRM17 and cTRM17 in the CD4⁺ compartment. These findings raise important questions regarding the triggers and regulatory mechanisms controlling retrograde migration.

It was previously suggested that secondary immune challenge induces tissue egress of antigen-specific TRM⁸⁹, which would be polarized towards the immune response the antigen usually induces. For example, immunization with vesicular stomatitis virus expressing ovalbumin induces OT-I CD8⁺ T cell-derived, antigen-specific TRM1 in mice. Antigen recall with ovalbumin peptide SIINFEKL in the skin generates antigen-specific CD103⁺ ex-TRM expressing CD49a⁸⁹, in line with a preferential TRM1 egress. CD4⁺ cTRM isolated from healthy human subjects on the other hand express high levels of CCR6 and CCR4 and produce IL-22 and IL-13, indicating ancestry from type 17- and type 2-polarized TRM⁹¹. Since these cells are associated with microbial control at steady-state^{227,228}, it is conceivable that

constant exposure of human skin TRM to microbes could mediate continuous low-level recall and tissue egress of mainly TRM17 in the absence of inflammation. While we confirmed increased IL-13 expression and found increased IL-17A expression of CD4⁺ cTRM compared to other blood T cells in aGVHD in study 1 (2.1.1.), we did not assess whether there is a difference between host and donor cTRM cytokine production in aGVHD, and whether cytokine levels differ compared to healthy controls. We also observed the expression of TRM1 marker CD49a on ~30% of CD8⁺ cTRM and ~ 25% among CD4⁺ cTRM following HSCT, where patients usually do not have ongoing skin inflammation. This suggests that retrograde migration is not only recall-dependent. It would be interesting to analyze whether increased host cTRM among total cTRM in aGVHD have a specific profile that might allow to differentiate between steady-state- vs inflammation- or recall-induced retrograde migration. Additionally, it should be clarified what is the contribution of bystander TRM to the antigen-specific ex-TRM pool in mice. Another potential confounder that remains elusive is variable retrograde migration potential of different tissue sites, caused for example by variable TRM density as well as the exposure to potential triggers across skin. This should be assessed in the future in preclinical models.

Furthermore, there are contradictory observations on the fate of ex-TRM in the circulation. While Fonseca et al report that SI intraepithelial lymphocyte TRM transdifferentiate into TCM in the blood⁸⁹, Behr et al found that secondary TRM-derived cells are mostly splenic TEM⁹⁰. This discrepancy might result from different methodologies and analysis timepoints. SI TRM that generate TCM are detected in the blood 100 days after secondary LCMV infection, as compared to SI TRM-derived TEM that are identified ~30 days after LCMV recall. To date, it is unclear how long an individual T cell is maintained in the blood after retrograde migration, as the earliest timepoints analyzed in different studies are two to three weeks. However, in both studies SI TRM keep the propensity to home back to the SI and are most efficient in generating secondary TRM, supporting the hypothesis that retrograde migration promotes the dissemination of tissue memory across large distances.

In our study (2.1.1.), we demonstrated that cTRM can co-express the gut homing marker integrin $\alpha 4\beta 7$, and CLA⁺ CD103⁺ T cells are present in GI GVHD lesions, suggesting that cTRM could re-seed at a distant organ site and thereby contribute to the dissemination of inflammation. However, we did not show formal proof for cTRM ancestry of gut T cells with this phenotype. Additionally, the markers currently used to identify cTRM in humans (CLA⁺, CD103⁺) likely underestimate the number of tissue-derived ex-TRM, since many TRM in humans are not expressing CD103. So far, we are lacking reliable tools to identify TRM progeny in the blood and addressing the fate of these cells remains challenging. Finding clonal overlaps between cTRM subsets and skin TRM subsets in high-dimensional datasets combining single-cell RNA and TCR sequencing would at least allow to make assumptions on likelihood of subset-specific retrograde migration and the tissue-traits cTRM retain. However,

since cTRM represent only a small fraction of blood T cells, with around 250-fold more skin T cells per cTRM distributed over a large body area⁹¹, finding clonal overlaps between cTRM and their skin counterparts is rare. Isolation of TRM from larger tissue pieces, for example from elective surgeries, combined with specific enrichment of cTRM from matched blood could likely provide more granular insights, but so far, such studies have not been performed. While it is clear, that some features of TRM are mirrored by cTRM/ex-TRM, this needs to be assessed carefully for each specific subset in a disease of interest, before these cells could be considered for biomarker studies in this disease. Overall, there is still a long way to go to define the specific mechanisms regulating retrograde migration.

Discordant observations on skin TRM heterogeneity in humans and mice

Heterogeneous TRM populations have been described in different contexts across tissues. In study 2 (2.2.1.) we detected host and donor TRM17 and TRM1 with distinct developmental and maintenance dynamics. While for the longest time it was thought that adaptation to a resident phenotype is relying on a common mechanism shared by these heterogeneous populations, recent studies in mice demonstrated that there are distinct molecular pathways used by different cells to acquire tissue residency⁷⁶. Mouse models examining mechanisms of residency have mostly been relying on infection or immunization models generating TRM1, leading to a biased view on *Hobit* as the main transcription factor required for residency. In mice, TRM17 are generated in the absence of inflammation after exposure to commensal microbes^{227,228}, and use a c-MAF-ICOS dependent axis for establishing residency⁷⁶. However, it is not clear how these findings translate to human TRM. While *ZNF683*, the human homologue of *Hobit* is expressed by human TRM, expression levels are low⁷⁹ and formal evidence for an absolute requirement of *ZNF683* for human T cell residency is lacking. Among human CD8⁺ skin TRM, Cheuk et al described a functional dichotomy of CD49a⁺ and CD49a⁻ cells, in which CD49a⁺ TRM are cytotoxic and produce IFN- γ , whereas CD49a⁻ produce IL-17a and express more *CCR6*⁷⁰, in line with TRM1 and TRM17 cells. These CD49a⁺ TRM depend on RUNX2 and RUNX3⁶⁹, suggesting that these transcription factors, rather than *ZNF683*, might govern human TRM1 development.

Importantly, in epigenetic profiles of CD49a⁻ TRM the most differentially accessible peak compared to CD49a⁺ TRM corresponds to *MAF*⁶⁹, the master transcription factor of murine TRM17. *MAF* was also highly expressed in the TRM17 cluster we identified in HSCT patients. In mice, TRM17 can be specifically depleted by targeting ICOS⁷⁶. Murine circulating CD4⁺ Th17 and Tfh cells express ICOS, and ICOS deficiency results in reduced expression of c-Maf and IL-21, limiting their maintenance²²⁹. This suggests that TRM17 may require continuous ICOS/ICOS-L signaling for sustaining c-Maf expression, as well as maintenance in the tissue. However, we did not observe high ICOS expression on TRM17 cells. Moreover, in the study by Cheuk et al, *MAF* but not *ICOS* was differentially expressed by CD49a⁻ compared to

CD49a+ epidermal TRM⁷⁰. Therefore, in human TRM17 other signals might be responsible for inducing c-Maf signaling. Interestingly, in LCMV infection models, ICOS/ICOS-L interactions of T cells and stromal cells were shown to be essential for the initial retention of TRM, enhancing *KLF2* downregulation and increasing efficient commitment to the TRM lineage²³⁰. Once residency is established, however, TRM do not require ICOS signaling for long term maintenance anymore²³⁰. Of note, this study did not distinguish between TRM1 and TRM17, although LCMV infection preferentially generates TRM1. So far, it is unclear how initial ICOS signaling in TRM1 and sustained ICOS signaling in TRM17 may be regulated, and if human TRM17 rely on ICOS at all. In the future, experiments in human TRM should clarify if TRM17 depend on continuous c-Maf expression and if so, which surface receptors besides ICOS could be engaged to transmit downstream c-Maf activation. Understanding these mechanisms will allow us to devise strategies to locally manipulate human TRM17 and potentially apply these strategies to restore tissue homeostasis in different skin diseases.

Two-faced role of TRM17 - pathogenic versus homeostatic

TRM17 are mediators of pathogenicity in several chronic inflammatory skin diseases. However, pathogenic TRM17 enriched in chronic inflammatory conditions might be distinct from homeostatic TRM17 present in healthy skin, as they usually acquire type 1 profiles. We observed a reduction of TRM17 in aGVHD lesions, with remaining TRM17 upregulating IFN- γ (2.2.1). Our observation can be explained by different scenarios: either existing homeostatic TRM17 lose lineage fidelity and repolarize to a TRM1-like phenotype, or they die and are replaced by newly generated TRM1 and pathogenic TRM17. aGVHD lesions in general are characterized by increased IFN- γ signaling and dominance of type 1 polarized T cells²³¹. Gene set enrichment analysis in GVHD returned terms related to type I and type II IFN signaling among the top hits across all skin cell types. Many genes in these pathways mediate suppression of type 17 polarization or development of pathogenic Th17/Tc17 cells.

For instance, in mice, pathogenic Th17 and Tc17 co-express IFN- γ and are highly proinflammatory^{207,232}. Responsiveness to IL-12 and co-expression of T-bet in Th17 cells has been linked to pathogenicity^{233,234}, and the latter can be induced by absence of TGF- β during differentiation²³⁴. TGF- β seems to be required for polarization of non-pathogenic Th17 cells, and is regulated by IRF1, IFN- β and BST2²³⁵. Fungi sensing by dectin-1 in human DC induces transient type I IFN responses, with tightly regulated expression levels of IFN- β through IRF1 and IRF5, leading to a controlled elevation of active TGF- β and non-pathogenic Th17 polarization²³⁵. Conversely, excess IFN- β induces BST2, which abrogates TGF- β activation²³⁵. *Ex vivo* profiling revealed that pathogenic Th17 express *IFNG*, *TBX21*, *IL1R1* and *CXCL3*, while homeostatic Th17 express *IL10*, *MAF* and *PTGDS*²³⁵. Of note, c-Maf cooperates with AhR for IL10 expression²³⁶, and *MAF*, *AHR* and *IL10RA* were downregulated by CD8 TRM in aGVHD in our study. In human granulomas, lesional Th17 express high amounts of *IFNG*,

CSF2 and *LTB* in cutaneous sarcoidosis¹⁶⁹, and in psoriasis CD8+ T cells co-express IFN- γ and IL-17A⁵⁶. *Ex vivo* stimulation of T cells from psoriatic lesions with IL-23 and IL-15 has a synergistic effect on IFN- γ and IL-17A production²³⁷, further indicating that a mixed type17/type1 signature characterizes these pathogenic cells.

How these signaling events impact on the developmental dichotomy of TRM1 and TRM17 regarding acquisition of residency remains unclear. In mice, *Tbx21* knockout significantly increases the generation of TRM17 compared to wildtype, suggesting that active T-bet signaling prevents formation of TRM17, as described for Th17 cells²³⁸. In our dataset, TRM downregulated *IL10RA* and *TGFB*, but upregulated *STAT1* and *IL12RB* in aGVHD, hinting at reduced c-Maf but upregulated T-bet signaling. Furthermore, myeloid and non-hematopoietic cells upregulated genes like *IRF1*, *BST2*, *STAT1*, *IL15* and *IL15RA*, pointing to an involvement of the tissue microenvironment in instructing TRM1 or homeostatic and pathogenic TRM17 fate decisions. Fate-mapping experiments in mice with established TRM1 and TRM17, subjected to chronic inflammation could help to clarify the mechanisms leading to pathogenic TRM17. In humans, in-depth computational analysis of various single-cell RNA sequencing datasets of different inflammatory lesions and healthy skin could provide new insights into divergent transcriptional profiles of pathogenic and homeostatic TRM.

Implications for HSCT and aGVHD research

Our findings challenge the graft-versus-host dogma of aGVHD pathogenesis. As described above, elevated host cTRM with pro-inflammatory properties hint at an additive role for host-vs-host or host-vs-graft immune reactions to graft-versus-host mediated pathogenesis. It may be beneficial to eliminate surviving host TRM before transplantation, to reduce the risk of activating these cells and unleashing their pro-inflammatory properties, both within the tissue and systemically by preventing retrograde migration and re-seeding of host cTRM. In mice, the presence of host TRM alone is sufficient to induce GVHD-like dermatitis when animals are transplanted with donor monocytes only¹⁴⁰. Disease severity in these animals is only mildly reduced compared to the PBMC-transplant group¹⁴⁰. In future pre-clinical studies, the role of radio-resistant host TRM should no longer be neglected, and adequate models accounting for their presence are required. As most mouse models use immunologically naïve mice, housed in specific-pathogen free facilities, they lack established TRM generated by previous immunological challenges. Developing models that consider host TRM-mediated effects will enable more efficient discovery of potential therapeutic targets, with higher chances of translatability to humans. Moreover, we require a better definition of the mechanisms conferring resistance to myeloablative conditioning to find vulnerabilities of host TRM and eventually be able to effectively deplete them.

Furthermore, our studies raise new questions about the controversial role of type 17 responses in aGVHD. While most studies support a detrimental role of Th17 and Tc17 cells,

they usually refer to pathogenic T cells, with IFN- γ co-production^{207,232}. However, there are reports that IL-17A alone can mediate protection from GI aGVHD²⁰³. Importantly, transferring the microbiome of IL-17 receptor deficient mice to wildtype mice also transfers susceptibility to GI aGVHD²³⁹. In the skin, we did not observe differences in IL-17A levels, but an upregulation of IFN- γ , which potentially overwrites the protective effects of IL-17A. While we did not provide experimental proof that acute loss of the TRM17 lineage directly contributes to aGVHD pathogenesis, it is likely that disbalanced T cell responses are detrimental. Cutaneous aGVHD lesions are characterized by an impaired skin barrier function and *Staphylococcus aureus* overgrowth¹⁶³, which could be a consequence of TRM17 reduction. Restoring homeostasis by normalizing the numbers and phenotype of TRM17 may be beneficial for aGVHD resolution. Whether this is the case, and how this could be achieved will need to be carefully examined in future studies and is not a trivial task. GVHD mouse models with the option to selectively deplete TRM1 or TRM17, by diphtheria toxin-induced depletion of Hobit-expressing cells or anti-ICOS administration, respectively, could serve as a starting point to address whether specific depletion of one or the other subset is beneficial for disease outcome. Co-culture experiments with human keratinocytes, *Staphylococcus* isolates from aGVHD lesions and TRM1 or TRM17 obtained from skin biopsies might be useful to reveal divergent roles in regulating host-microbiome interactions and barrier integrity.

Furthermore, it should be assessed whether depletion of TRM17, as well as CD8 cTRM17, precedes clinical aGVHD development. If so, especially abundance of CD8 cTRM17 in peripheral blood could serve as a biomarker for aGVHD onset. However, this will require multicenter prospective studies with large numbers of patients that can account for clinical parameters such as conditioning regimen, aGVHD prophylaxis and degree of matching, as well as age, sex and other potential confounders.

Targeted manipulation of tissue TRM for precision medicine approaches

As I described in the introduction, TRM are involved in every type of immune response and are present in virtually all non-lymphoid organs. Therefore, the site- and subset-specific manipulation of TRM harbors great potential for precision medicine approaches, not only in the context of GVHD. For example, finding ways to prevent the switch from homeostatic to pathogenic skin TRM17 cells could be beneficial in diseases like psoriasis or sarcoidosis.

In vaccine design, there is growing appreciation for differential vaccination efficacy and long-term protection depending on the delivery routes, which depend on the generation of TRM in that tissue (reviewed by Rotrosen & Kupper²⁴⁰).

Dissecting tissue-specific mechanisms of TRM recruitment and maintenance can also guide more efficient cell therapy approaches. So far, the broad application of chimeric antigen receptor (CAR) T cell therapy in solid cancers has been hampered by the limited efficacy of CAR T cell recruitment and maintenance into peripheral tissues. However, recent results

indicate that CAR T cells endowed with TRM features more efficiently control solid tumors²⁴¹. In addition to cancer treatment, B cell targeting anti-CD19 CAR T cell therapy has also shown promising results in autoimmune diseases, such as systemic lupus erythematosus²⁴². In mice, repurposing CAR T cells to target autoreactive cells was achieved in a model of pemphigus vulgaris, by engineering CAR T cells expressing an autoantigen, desmoglein 3, fused to conventional CAR signaling domains²⁴³. These cells specifically eliminated autoreactive B cells expressing desmoglein 3-specific B cell receptors *in vivo*²⁴³. Currently a phase 1/2 open-label clinical trial assesses the safety and optimal dosing in pemphigus vulgaris patients (NCT04422912). These kinds of therapies could benefit from more precise engineering of cell therapy products, to improve on-target and reduce off-target effects. In the future, one could envision precise CAR T cell design for autoimmune tissue diseases and solid cancers. In such an approach, CAR T cells could be tailored to specifically reach the tissue of interest and establish residency there, while at the same time targeting and eliminating pathogenic immune cells or cancer cells specifically in this tissue. Therefore, studying human TRM in their tissue context, and meticulously dissecting their specific phenotypes and functions is important to accurately predict their behavior in organ-specific diseases and come up with targeted therapeutic approaches.

Prospects for a comprehensive understanding of human tissue immunity

The advance of single-cell RNA profiling technologies has started a new era of research on human tissues. High-throughput sequencing methods empower in-depth analysis of cellular phenotypes and return data with high information density, even from small biopsies. The Human Cell Atlas (HCA) as a global initiative aims to map every single cell in the human body across age and different disease settings by collecting and integrating large multi-omics datasets with single-cell resolution. Currently, single-cell RNA sequencing data from more than 61 Million cells across different organs from 9100 donors and 475 projects are accessible on the HCA website²⁴⁴, enabling open access for researchers worldwide.

Lately, also spatial transcriptomics techniques achieve subcellular resolution, which will be instrumental to understand the spatial context of phenotypically and functionally diverse cells in a tissue. For example, in a recent preprint by Reina-Campos et al, spatial transcriptomics of the SI revealed a preferential location of effector-like TRM at the villus tip, while memory-like TRM reside in the crypt²⁴⁵. Including spatial information will also drastically improve the predictive power of computational tools inferring cell communication based on ligand-receptor expression²⁴⁶. Platforms like open-ST or Nova-ST even enable 3D reconstruction of whole tissue biopsies at relatively little cost^{247,248}. The major challenge is to integrate datasets across different donors, tissues, modalities and technical protocols, to extract biologically meaningful information from this data and to distinguish it from batch effects and noise. This requires

streamlined bioinformatic analysis pipelines and strict quality control steps at all stages of analysis.

Another important aspect is to establish frameworks facilitating research on human tissue immunity across organs. Especially obtaining tissue samples from internal organs of healthy human donors remains a challenge. One valuable source for this is collection of organs from brain-dead individuals. For example, collaborations of organ procurement organizations and research labs enable the simultaneous sampling of matched tissues, after organs designated for transplantation have been collected²⁴⁹. Analysis of such samples has yielded important insights into heterogeneity of anti-viral responses in different tissues²⁵⁰, site-specific adaptation of TRM⁶⁵, and distinct functions of barrier-site TRM in early life⁷⁵. The coordinator of these studies recommended that for advancing work on human tissue immunity, researchers and clinicians should promote the paired sampling of different tissues from individual patients, modify sample collection protocols for biobanks to include frozen tissues containing viable immune cells and foster collaborations between clinics and basic research departments²⁴⁹.

Overall, researchers have the tools at hand to reconstruct immunological processes in human tissues with unprecedented detail. Comparing these processes across organs and diseases will enable the discovery of new treatment targets, with hopefully high rates of clinical translatability.

3.2. Conclusions and outlook

Since the discovery of TRM in the mid-2000s, seminal studies about their biology have tremendously increased our understanding of protective and pathogenic immunity in barrier tissues. My thesis has contributed to the field of TRM research by highlighting their potential for retrograde migration and systemic spreading to distant barrier sites, and by shedding light on their functional heterogeneity, adaptive plasticity and their potentially distinct developmental dependencies. In the future, I want to investigate the contribution of cell signaling from the tissue microenvironment to TRM phenotypes in aGVHD. Specifically, I would like to explore how structural cells, such as fibroblasts and keratinocytes, instruct the maintenance and polarization of human TRM, and how this cellular crosstalk changes during inflammation.

4. Materials & Methods

Detailed descriptions of materials and methods are included in the individual manuscripts in the respective “Material and Methods” sections, for 2.1.1. pages 41-43 of this thesis and for 2.2.1 pages 70-78.

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Appendix

Review of chronic Graft-versus-Host Disease

As mentioned in the introduction, pathophysiological mechanisms mediated by immune cells during the chronic form of GVHD (cGVHD) were not described in this thesis, because in my project I mainly worked with aGVHD patient samples and because I published a first-author review on this topic. In this review, which is included here, the aim was to highlight the specific pro- and anti-inflammatory signals of distinct immune cell subsets during cGVHD.



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Complex interactions of cellular players in chronic Graft-versus-Host Disease

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Chronic Graft-versus-Host Disease is a life-threatening inflammatory condition that affects many patients after allogeneic hematopoietic stem cell transplantation. Although we have made substantial progress in understanding disease pathogenesis and the role of specific immune cell subsets, treatment options are still limited. To date, we lack a global understanding of the interplay between the different cellular players involved, in the affected tissues and at different stages of disease development and progression. In this review we summarize our current knowledge on pathogenic and protective mechanisms elicited by the major involved immune subsets, being T cells, B cells, NK cells and antigen presenting cells, as well as the microbiome, with a special focus on intercellular communication of these cell types via extracellular vesicles as up-and-coming fields in chronic Graft-versus-Host Disease research. Lastly, we discuss the importance of understanding systemic and local aberrant cell communication during disease for defining better biomarkers and therapeutic targets, eventually enabling the design of personalized treatment schemes.

KEYWORDS

chronic graft-versus-host disease, hematopoietic stem cell transplantation, immune cell networks, cell-cell communication, GvHD pathogenesis

Introduction

For patients with hematologic malignancies, primary immunodeficiencies or bone marrow (BM) failure, allogeneic hematopoietic stem cell transfer (HSCT) is often the only option for curative treatment. This treatment, however, comes with a high risk of developing acute or chronic Graft-versus-Host Disease (GVHD). While acute GVHD

(aGVHD) occurs early after transplantation and mainly affects the skin, liver and gut, chronic GVHD (cGVHD) establishes later after transplantation and is a heterogeneous syndrome with multi-organ involvement. Patients typically require long-term treatment with immunosuppressive drugs and are faced with a high risk of mortality and severe impact on their quality of life. The pathogenesis of cGVHD involves multiple processes, which are complex and inter-dependent. Recently, a 3-phase model for cGVHD was proposed that involves early inflammation and tissue injury including thymic damage with loss of central and peripheral tolerance, subsequent chronic inflammation and dysregulated immunity and eventually aberrant tissue repair with fibrosis (1).

A diverse set of immune cellular players mediate cGVHD

Every phase of cGVHD involves a diverse set of immune cells, which exert several disease-promoting pathomechanisms. The precise interplay between these cell types and mechanisms on a systemic level as well as in the affected tissues is incompletely understood. In this review we give an overview of the different immune components involved in cGVHD pathogenesis and summarize our current knowledge about dysregulated cell composition, signaling and intercellular communication in cGVHD. Specifically, we address mechanisms and therapeutic potential of T cell regulated pathogenic signaling and give an overview of disturbances in the B cell compartment. We discuss the dual role of NK cells, touch upon the role of mixed chimerism of antigen presenting cells (APCs), macrophage polarization and structural cells as APC in disease pathogenesis, and outline immunomodulatory mechanisms mediated by the gastrointestinal microbiome in cGVHD.

Cellular crosstalk in cGVHD via extracellular vesicles – a new field of GVHD research

The different cell types involved in disease pathogenesis do not act as single entities but are communicating with each other to facilitate their effector functions. Extracellular vesicles (EVs) play a central role in immune cell crosstalk and represent an entirely new area for research in the context of GVHD.

All eukaryotic cells release EVs that contain a subset of proteins, lipids and nucleic acids, predominantly small RNA (smRNA), that are derived from the parent cell. These particles vary in generation method and size, including shedding microvesicles (1000–100nm) and exosomes (150–30nm) from multivesiculated bodies generated in the endocytic pathway. Therefore, EVs are heterogeneous and differ in their content and surface proteins. It is now well established that EVs play an important role in intercellular communication by transferring their contents between cells (2). They are also involved in numerous essential physiological processes, and vesicles from both non-immune and immune cells have important roles in

immune regulation (3). EVs play a role in both innate (4) and adaptive immunity where they can have both pro- and anti-inflammatory capabilities, depending on their contents. They can serve as disease biomarkers for aGVHD, as recently shown by Lia et al (5), but are likely involved in several GVHD promoting mechanisms by facilitating cell-cell communication. EVs in GVHD have so far mostly been studied in the context of mesenchymal stem cell (MSC)-based therapy. Several mouse models and few studies in human subjects revealed that the immunomodulatory properties of MSC are largely mediated by their secretome, which includes EVs. These effects have recently been extensively reviewed in Doglio et al (6) and Lia et al (7). However, also immune cells themselves are releasing EVs that have the potential to exert autocrine and paracrine immunomodulatory effects. These mechanisms are thus far understudied in GVHD. In the following chapters we summarize recent findings regarding EVs produced by a variety of immune cells which are known to be involved in the etiology of GVHD, discuss their effects and how they could shape the inflammatory environment during active GVHD.

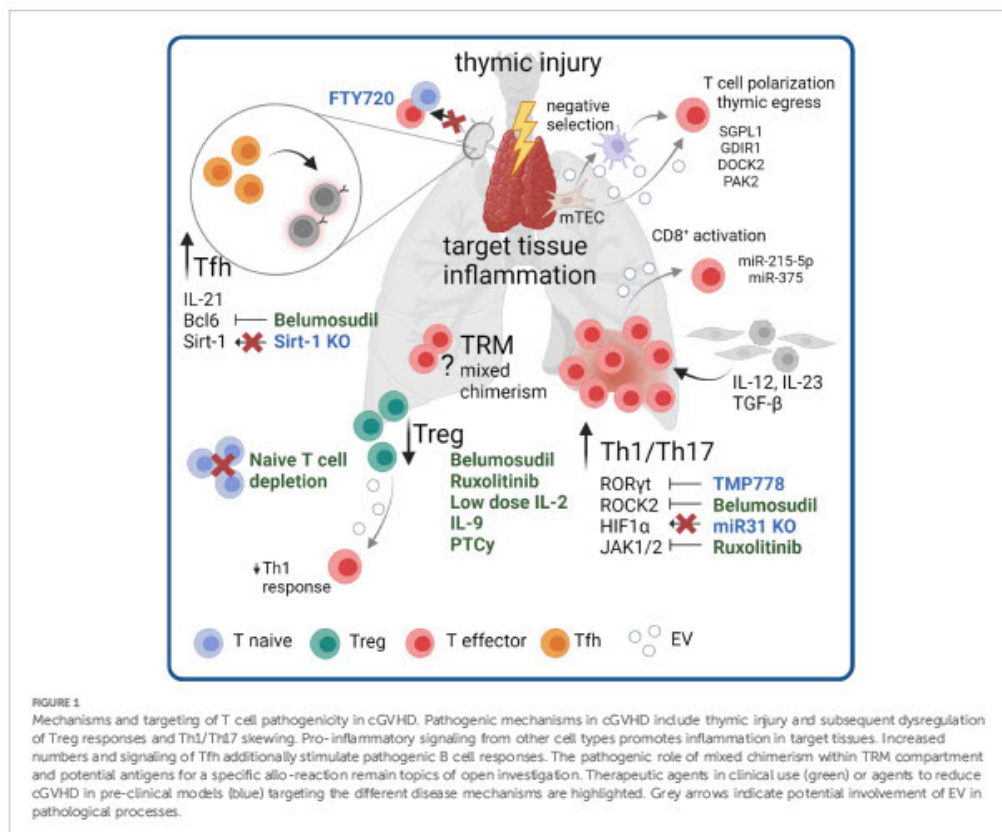
The role of EVs in the pathophysiology of immunological disorders is an up-and-coming field for research, as they offer translational importance as prospective therapeutic targets, informative biological agents, and predictive disease biomarkers.

Novel mechanisms of T cell pathogenicity in cGVHD

The pathophysiology of cGVHD is complex and involves a plethora of different organ systems and cell types, and both innate and adaptive immune responses are involved. Especially in the second phase of cGVHD development with chronic inflammation and dysregulated immunity, adaptive immune responses are initiated and result in a loss of central and peripheral tolerance and the emergence of pathogenic T cells and B cells, causing auto- and alloimmune reactions. In this chapter we highlight novel players identified in pathogenic T cell signaling in cGVHD and how communication *via* EVs could contribute to pathogenic effects (Figure 1).

Th1/Th17 polarized T cells dominate inflammatory responses in cGVHD

Different subsets of CD4⁺ T cells have been reported to have crucial roles in disease development and maintenance. Principally, cGVHD is dominated by Th1/Th17-like immune responses, with a simultaneous loss of Treg function. Thymic injury during disease progression leads to diminished Treg generation and release of autoreactive T cells becoming IL-17A producing effectors (reviewed in (8)). Recently, Alawam et al. reported a failed recovery of medullary thymic epithelial cells as the main mechanism for impaired Treg development and escape of self-reactive conventional $\alpha\beta$ T cells in mice after syngeneic BM transplantation (9). T cells with Th1/Th17 polarization are enriched in cGVHD target tissues such as the skin and liver (10, 11), and are thought to mediate



chronic inflammation. The Th1/Th17 skewing in cGVHD target tissues is driven by mediators produced by other immune and stromal cells within these tissues. Thereby, the IL-12 cytokine family plays an important role, as IL-12 and IL-23 are the major drivers of Th1 polarization and Th17 maintenance (reviewed in (12)). The main cytokine sources are activated macrophages and dendritic cells in tissues, although IL-23 production by keratinocytes has been described in the context of skin inflammation (13, 14). Keratinocytes and other epithelial cells produce TGF- β , an important cytokine for Th17 as well as Treg polarization, wherein the former additionally requires the presence of IL-6 or IL-21 (15). IL-6 can be produced by many stromal and immune cell types, whereas IL-21 production is restricted to activated T cells.

Recently, Forcade et al. showed that cGVHD patients represent with elevated frequencies of CD146+ CCR5+ T cells, which have enhanced migration potential and Th1/Th17 features (16). The same study could show that TMP778, a small molecule inhibiting Th17 master transcription factor ROR γ t, alleviated murine cGVHD (16). Approaches to target Th1/Th17 cells to prevent or revert cGVHD have become a topic of increasing interest. A recent mouse study revealed a role for microRNA (miR) -31 in T cell

pathogenicity in cGVHD. miR-31 deficient T cells displayed reduced Th17 differentiation and increased Treg generation through loss of HIF1 α upregulation in allogeneic cells, leading to improved cutaneous and pulmonary cGVHD (17). Of note, HIF1 α signaling is known to boost Th17 skewing in T cells and can be induced by TCR signaling upon target cells recognition, cytokine signaling, or hypoxia, which is a hallmark of inflamed tissues (reviewed in (18)). Pre-clinical findings show that ROCK2 inhibition reduces production of Th17 signature cytokines IL-17 and IL-21 in a STAT3-dependent manner, while increasing STAT5 signaling, thereby promoting Treg function (19, 20), have led to clinical studies testing ROCK2 inhibitors in GVHD, which we discuss later in this chapter.

Dysregulated regulatory T cells prompt failed tolerance

Dysregulated Treg immunity is another hallmark feature of cGVHD (21, 22). Failure to properly reconstitute the Treg compartment after HSCT results in loss of tolerance, allo- and

autoimmunity and impaired control of immune responses (21, 22). Importantly, Treg expand in secondary lymphoid organs and subsequently migrate to peripheral tissues, where they suppress local inflammatory responses and prevent the early expansion of alloreactive effector cells (23, 24). Low-dose IL-2 therapy for Treg induction has shown beneficial effects on Treg numbers in cGVHD, albeit a considerable number of patients are unresponsive (25–27). A recent study demonstrated preferential activation of effector T cells over Tregs in a severely inflammatory environment and therefore suggested IL-2 treatment to be tailored to the immune status of each patient (28). Nicholls et al. investigated BM-residing Tregs (BM-Treg) in the context of cGVHD, which failed to reconstitute properly and did not expand in response to IL-2 but rather IL-9. Importantly, BM-Treg depletion, while maintaining splenic Treg, resulted in exacerbated skin cGVHD (29). Type 1 adaptive regulatory cells (Tr1) are another type of Treg, characterized by absence of Foxp3 expression, high IL-10 production, and co-expression of CD49b and LAG-3 (30), and are crucial to control aGVHD in mice (31). In human cGVHD, the Tr1 subset was shown to be specifically enriched in patients with active disease compared to patients in remission, whereas the inverse was true for thymic Treg (32). However, the functional role of Tr1 in disease control needs to be further addressed. Over the past years post-transplant cyclophosphamide (PTCy) has emerged as a promising and effective treatment strategy to reduce the incidence of cGVHD. Although initially the effect was attributed to a general impairment of allo-reactive donor T cells, newer studies found a Treg-specific sparing effect (33–35). Comparing T cells in patient blood receiving PTCy with no PTCy regimens revealed a significantly delayed T cell reconstitution with a simultaneous increase in the proportion of Treg in the PTCy group (35). PTCy patients also displayed a higher level of inhibitory markers on both CD4⁺ and CD8⁺ cells, with reduced capacity for cytokine production after ex vivo stimulation, giving new insights into the mechanism of action of PTCy-mediated cGVHD prevention.

Follicular helper T cells enable B cell pathogenicity

Another T cell subset critically implicated in cGVHD pathogenesis are follicular helper T cells (T_{fh}), a subset of CD4⁺ T cells located in the B cell follicle and expressing the transcription factor Bcl6 along with high levels of the chemokine receptor CXCR5 and programmed cell death protein-1 (PD-1). T_{fh} cells are crucial for initiating B cell-mediated disease mechanisms by enabling germinal center formation through IL-21 production, and increased T_{fh} numbers correlate with disease severity (36). ROCK2 inhibition in murine cGVHD, in addition to regulating the Th17/Treg balance, decreased the T_{fh} master transcription factor Bcl6 and consequently the frequency of T_{fh} (19). Another mouse study proposes Sirt-1 as a new cGVHD target, as T cell-specific Sirt-1 deficiency negatively affected T_{fh} generation, B cell activation and plasma cell differentiation, while simultaneously promoting Treg stability (37). Similarly, IL-39, a recently described IL-12 family cytokine,

aggravated cGVHD in mice by promoting STAT1/STAT3-mediated CD4⁺ T cell activation and differentiation of germinal center B cells in mice after allogeneic (allo-)HSCT (38). This study described activated B cells, CD11b⁺ cells and CD8⁺ T cells as main IL-39 sources. However, there is so far no evidence for a pathogenic function of IL-39 in human GVHD.

T cell targeting strategies are promising new therapeutic

The overall importance of dysregulated T cell activation and polarization of T cell subsets in cGVHD pathogenesis is underlined by the therapeutic efficacy of Ruxolitinib, a selective Janus kinase (JAK) 1/2 inhibitor. Blocking JAK/STAT signaling affects different aspects of T cell biology related to cGVHD pathogenesis, such as production of pro-inflammatory cytokines, proliferation and survival and the differentiation into IFN- γ and IL-17A-producing cells (39). In September 2021, the FDA approved Ruxolitinib for cGVHD after failure of one or two lines of systemic therapy in adults and children >12 years, based on results of the REACH-3 (NCT03112603) trial (40). Additionally, the selective JAK1 inhibitor Itacitinib in combination with corticosteroids is currently tested as first-line therapy for cGVHD (NCT03584516). As mentioned above, promising results for ROCK2 inhibition were obtained in pre-clinical models. Belumosudil, a selective ROCK2 antagonist, was tested for safety, tolerability, and activity in cGVHD patients in clinical studies (NCT02841995, NCT0344081) (41, 42). These studies included heavily pre-treated refractory patients having failed one or more previous lines of therapy and reported overall response rates (ORR) of > 70% (41) and > 60% (42), respectively, in all treatment groups, with limited cytotoxicity and improved quality-of-life. These results suggest Belumosudil therapy to be suitable for patients with treatment-refractory cGVHD, which resulted in FDA approval for cGVHD patients 12 years or older after failure of two or more prior systemic treatments in July 2021 (43).

Additional attempts to prevent the dissemination of allo- and autoreactive T cells include the depletion of naïve T cells before transplantation and blocking of T cell migration. Since mouse studies demonstrated exacerbated disease mediated by naïve T cells compared to memory T cells (44, 45), and naïve T cell numbers correlate with the later onset of cGVHD and are elevated in starting cGVHD in patients (46), clinical studies on the efficacy of naïve T cell depletion from the graft were conducted. Recently published results from three clinical trials (NCT00914940, NCT01858740, NCT02220985) revealed a very low rate of cGVHD in those patients (47). From 138 patients, 1% developed moderate-severe cGVHD and the 3-year GVHD-free, relapse-free survival was 68%, suggesting naïve T cell depletion could be a novel superior strategy of graft engineering (47). In mice, application of the S1P agonist FTY720, which blocks lymphocyte migration and traps naïve and effector T cells in lymph nodes, reduced cGVHD severity and fibrosis (48). Of note, according to a case report by Gauthier et al., a patient with severe central nervous system GVHD was successfully treated with FTY720 (49).

Tissue T cell chimerism can drive inflammation

So far, efforts to uncover T cell pathogenicity in GVHD have focused on donor T cells. However, it has lately become evident that host-derived tissue-resident memory T cells (TRM) can survive the conditioning regimen and persist in GVHD target tissues, such as the skin and the gut (50–52). The presence of host TRM in other GVHD target tissues has thus far not been reported but is likely due to the high abundance of TRM in tissues such as liver and lung (53, 54). In patients' skin with active GVHD, these host-derived T cells were proliferative and produced effector cytokines (51, 52). Data obtained in a cutaneous GVHD humanized mouse model demonstrated that host TRM activated by donor APC are sufficient to induce a GVHD-like dermatitis (51). Host T cells with a resident phenotype could be found in increased numbers in the blood of aGVHD patients and were able to mediate keratinocyte damage in an *ex vivo* model (55). On the other hand, in murine GVHD models, mice receiving T cell depleted bone marrow usually don't develop skin manifestation. This discrepancy may result from species differences in tissue resident T cell biology, and artifacts such as low abundance of antigen experienced TRM in laboratory mice compared to humans or pet store "dirty" mice (56). In fact, patients receiving T cell depleted PBSCT can still develop cutaneous aGVHD (57), underlining the importance to study disease mechanisms in human models to translate pre-clinical findings.

Host TRM in the skin were reported to have low replacement rates and persist in the skin at ranges of 5–95% 10 years after transplantation (52). Another study reports that 23% of patients display long-term skin T cell chimerism (50). Interestingly, in this study skin T cell chimerism occurred in 57% of patients with aGVHD, but only 12.5% of patients with cGVHD. Importantly, these studies demonstrated that a previous history of GVHD correlated with a loss of host TRM, indicating that host TRM might be preferentially targeted by infiltrating alloreactive donor T cells (50, 51). These data suggest that host TRM, while being dispensable for GVHD development, can contribute to GVHD related inflammation. However, the specific pathogenic mechanisms, and especially a potential role of host TRM in cGVHD pathology, must be further investigated. In the future, the development and use of TRM specific depletion models for specific organs should give clearer insights into the role of these cells in aGVHD and cGVHD.

EVs mediate T cell pathogenic signaling

EVs play a role in the development of T cells. Thymic epithelial cells have been shown to release EVs containing tissue restricted antigens (58) which are then presented to thymic cDCs for antigen presentation, contributing to the negative selection of T cells with specificity to self-antigens (58). In the context of cGVHD, this may have an important effect on the maintenance of central tolerance,

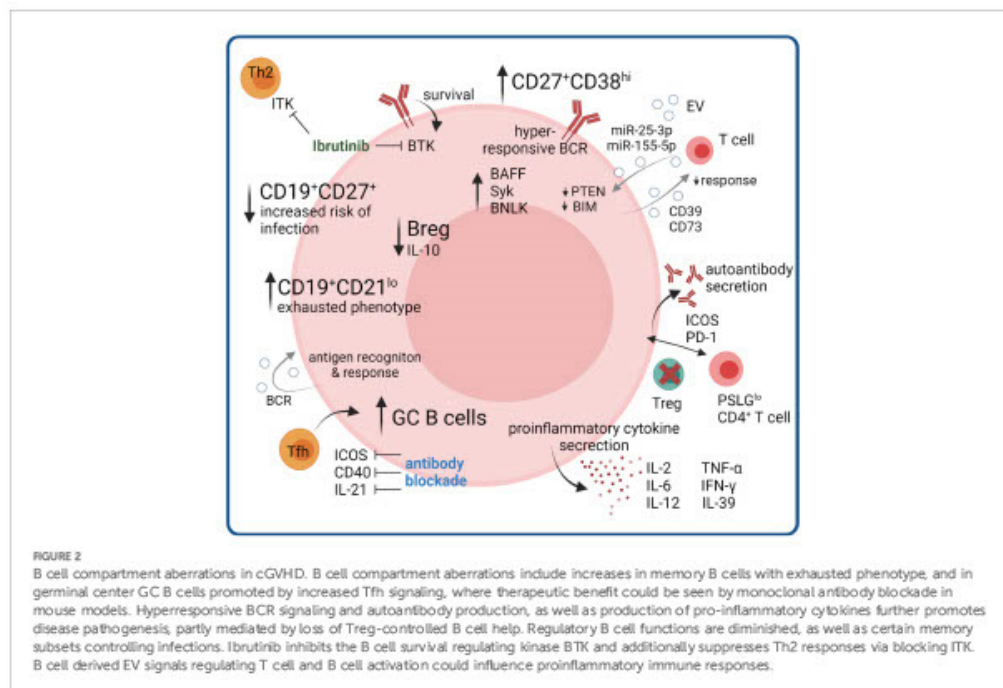
due to potential changes in the type and content of EVs during thymic damage. More specifically, EVs containing molecules such as sphingosine-1-phosphatase lyase 1 (SGPL1), Rho GDP-dissociation inhibitor 1 (GDIR1), dedicator of cytokinesis protein 2 (DOCK2), and p21 protein-activated kinase 2 (PAK2) contribute to the polarization of CD4 or CD8 single positive thymocytes, aiding both maturation of T cells and thymic egress, but continue to contribute to T cell function in the periphery (59).

The ability of Tregs to enforce peripheral tolerance, and suppress Th1 cell responses, has been shown to be partly *via* nonautonomous gene silencing, mediated by miRNA-containing EVs (60). Indeed, Tregs are prolific producers of EVs that contain high levels of miRNA, the profile of which is distinct from those of Th1 and Th2 cells. Treg derived EVs can transfer specific sets of miRNA to conventional T cells, both *in vitro* and *in vivo* (60, 61). Compromised transfer of Treg EV miRNAs to conventional T cells (either *via* failed miRNA formation (Treg cell Dicer deficiency), or inhibited EV release (Rab27a- and Rab27b-deficient Tregs) has been shown to abrogate the capacity of Tregs to prevent disease in a colitis model, by specifically regulating Th1 cell responses (60). The specific role of Treg EV mRNA and proteins in modulating target cells in a context-dependent manner remains unknown, but seems likely to drive their immunomodulatory effects. Loss of Tregs and their tolerogenic function enables pro-inflammatory immune cells to perpetuate GVHD.

Overall, T cells remain the most studied immune cell subset in both aGVHD and cGVHD, as they are crucial for disease development and maintenance and the most obvious target for preventive and curative therapies. However, many studies focus on the blood, studying systemic disease effects. With our improved understanding of tissue immunity and the diverse phenotypes T cells can acquire in different organs, there is a need to investigate tissue-specific disease mechanisms. A recent study in a murine GVHD model demonstrated that disease is maintained in target organs solely by resident T cells and their progenitors (62), highlighting the need to develop tissue-targeting therapies.

Communication of immune cells in cGVHD – the role of B cells

The lymphoid system is known to be a target organ of cGVHD seen in lymphoid hypocellularity, functional asplenia and humoral immune deficiency (63–65). B cells have a substantiated role in cGVHD including dysregulation of the B cell compartment, aberrant B cell signaling pathways, production of allo- and autoantibodies and T – B cell interactions. Aberrant B cell homeostasis with reduced generation of BM B lymphoid progenitors (65–68), low frequencies of naïve and memory B cells (69, 70) and a regulatory B cell defect have been observed in cGVHD (Figure 2) (71). Here, we will give an overview of aberrant B cell subpopulations and B cell signaling in cGVHD and highlight abnormalities in T and B cell crosstalk.



Aberrant B cell subpopulations and signaling pathways characterize cGVHD

Significantly higher relative numbers of CD19⁺CD21^{low} B cells in patients with long-lasting or first diagnosis of cGVHD were reported and this B cell subpopulation significantly correlated with activity and severity of cGVHD (46, 69, 70, 72–75), respectively. In addition, CD19⁺CD21^{low} B cells were elevated on day +100 after allo-HSCT in patients subsequently developing cGVHD (46). Khoder et al. further characterized the CD19⁺CD21^{low} B cell subset demonstrating features of exhaustion, such as increased expression of multiple inhibitory receptors, altered expression of chemokine and adhesion molecules and poor proliferative response to a variety of stimuli (72). Further research with modern single-cell approaches combining antigen specificity and phenotype will elucidate whether the exhaustion results from constant antigen exposure or from of unspecific activation. It is also unclear whether exhausted B cells affect cGVHD pathogenesis, are a mere consequence of persistent inflammation or a cause of defective B cell reconstitution. A recent single cell study reported the expansion of CD27⁺ memory and CD11c⁺ atypical memory B cells in cGVHD patients, which can exhibit an exhausted phenotype. Trajectory analysis predicted that the cGVHD-expanded cluster develops along different trajectories compared to corresponding healthy control populations, bypassing tolerance checkpoints (76).

Both B cell receptor (BCR) signaling and B cell activation factor (BAFF) play key roles in determining B cell fate and survival.

Multiple groups have shown that cGVHD is closely associated with aberrant BAFF levels (46, 67, 74, 77, 78), an activated B cell phenotype, and aberrant BAFF/B cell ratios (46, 67, 74, 78, 79). Excessive BAFF levels have been associated with increased proportions of CD27⁺ B cells, including both pre-germinal center (GC) CD27⁺CD38^{hi}IgD⁺ and post-GC CD27⁺CD38^{hi}IgD⁺ B cells in patients with active cGVHD as compared to healthy individuals (78). B cells from cGVHD patients had significantly increased proliferative responses to B cell receptor (BCR) stimulation and resistance to apoptosis, resulting in the constant activation of B cells (80–82). BCR hyperresponsiveness is reportedly associated with increased expression of proximal signaling molecules including spleen tyrosine kinase (Syk) and B cell linker (BLNK) protein (81), an effect known to be mediated *via* increased BAFF levels. Activated CD27⁺ B cells from cGVHD patients are in an increased metabolic state with bigger size and protein content and are primed for survival *via* BAFF pathways (80). The Syk inhibitor Fostamatinib was recently tested in a Phase I clinical trial (NCT02611063) for cGVHD prophylaxis and as steroid-refractory cGVHD treatment (83). In both arms the proportion of post-GC plasmablasts like B cells decreased in the early treatment phase (83). Bruton's tyrosine kinase (BTK) is downstream of the BCR and required for tonic and antigen-dependent receptor signaling which regulates B cell survival (84). Ibrutinib, an FDA-approved drug for steroid-refractory cGVHD inhibits BTK in B cells, leading to reduced production of autoreactive antibodies but also inhibits a homologous enzyme in T cells, interleukin-2-inducible T cell kinase

(ITK), leading to selective suppression of Th2 immune responses that may contribute to the pathogenesis of cGVHD (85, 86). However, recent results from the INTEGRATE study (NCT02959944), evaluating first-line treatment of cGVHD with iburitinib-prednisone showed no benefit compared to placebo-prednisone treatment, which may be attributed to different patient cohorts and study design or reflect different immune mechanisms active in steroid-refractory compared to untreated cGVHD (87). In mice with cGVHD combinatorial treatment with BTK and ITK was beneficial (88), warranting further studies to explore the benefit of combination therapy in humans.

Another hallmark of cGVHD is the lower frequency of CD19⁺CD27⁺ memory B cells (69, 70), whose reduced number is associated with the incidence of infectious complications after allo-HSCT (69, 70, 74). Of note, resolution of cGVHD correlated with expansion of CD19⁺CD27⁺ memory B cells (69). Using B cell rearrangement excision circle measurements, Glauzy et al. observed increased B cell replication but decreased overall B cell neogenesis in patients with aGVHD and cGVHD (67).

B cells can have regulatory properties that suppress rather than initiate cGVHD, and regulatory B (Breg) cells have been identified as a novel B cell subpopulation associated with cGVHD development (71, 89, 90). Compared to healthy individuals, cGVHD patients have decreased Breg cells defined as interleukin (IL)-10 secreting cells with reportedly different phenotypes. Khoder et al. defined B cells with immunoregulatory properties within both the CD19⁺IgM⁺CD27⁺ memory and CD19⁺CD24^{high}CD38^{high} transitional B cell compartments and observed a lower frequency of Breg cells in patients with cGVHD (89). Furthermore, Breg cells in these patients were less likely to produce IL-10 compared to Bregs from healthy donors. De Masson et al. observed enriched IL-10 production in both the CD24^{hi}CD27⁺ and CD27^{hi}CD38^{hi} plasmablast B cell compartments (71). Patients with cGVHD had less CD24^{hi}CD27⁺ B cells and IL-10-producing CD24^{hi}CD27⁺ B cells and increased CD27^{hi}CD38^{hi} plasmablast frequencies, but decreased IL-10-producing plasmablasts (71).

Abnormal T and B cell interactions characterize cGVHD

Antigen presentation by activated B cells that have up-regulated major histocompatibility complex (MHC) and costimulatory molecules, such as CD80 and CD86, leads to CD4⁺ and CD8⁺ T cell activation and differentiation. Depending on the B cell subpopulation and the nature of the activation stimulus, B cells can also act as immunoregulatory cells that induce peripheral CD4⁺ and CD8⁺ T cell tolerance, inhibit dendritic cells, and induce and expand regulatory T (Treg) cells.

Amongst other mechanisms, cGVHD is mediated by abnormal CD4⁺ T and B cell interactions, due to the lack of donor-derived Treg cells (91, 92). B cells contribute to immune responses by antibody-mediated and antibody-independent mechanisms. Antibodies directed against Y-chromosome-encoded epitopes

(H-Y antibodies) in male recipients of stem cell grafts from female donors developing cGVHD have been observed (93–95). The presence of auto-antibody-secreting B cells promoted by alloreactive donor CD4⁺ T cells is an important mediator of autoimmune features of cGVHD, suggesting that a loss of B cell tolerance is operative (92, 96, 97). A number of autoantibodies, including anti-nuclear antibodies (ANAs), anti-double-stranded DNA (anti-dsDNA), and anti-platelet-derived growth factor receptor (PDGFR)- α , have been found in association with cGVHD (98–102).

In a preclinical cGVHD model, B cells were required to induce cGVHD and associated bronchiolitis obliterans syndrome (BOS) (103). Of note, tissue fibrosis was observed only after secretion of high-affinity class-switched antibodies that activate immune responses and, in the case of cGVHD, cause severe damage to target tissues by activating complement or antibody-dependent cell-mediated cytotoxicity. Furthermore, in this well-established murine cGVHD model, donor T cell-derived Th cells were required for the activation of donor BM-derived B cells that differentiate into GC B cells, resulting in immunoglobulin deposition in BOS lesional tissue (104). Increased frequency of Th cells correlated with increased GC B cells, cGVHD, and BOS. Development of BOS was dependent upon T cells expressing the chemokine receptor CXCR5, to facilitate T cell trafficking to secondary lymphoid organ follicles. Th cells support the generation of GCs by providing signaling through IL-21, inducible T-cell co-stimulator (ICOS), and CD40 (105–108); blocking these factors by monoclonal antibodies hindered GC formation and cGVHD (104).

In a murine model, Deng et al. observed that cGVHD causes destruction of lymphoid follicles and absence of GCs in lymphoid tissues (109). Development of cGVHD did not require GC formation and was associated with the expansion of extrafollicular CXCR5⁺BCL6⁺PSGL1^{low}CD4⁺ T cells in GVHD target tissues (109). The same research group demonstrated that murine and human PSGL1^{low}CD4⁺ T cells from GVHD target tissues have features of B cell helpers with upregulated expression of PD1 and ICOS and production of IL-21 (110). Murine PSGL1^{low}CD4⁺ T cells from GVHD target tissues augmented B cell differentiation into plasma cells and production of autoantibodies via their PD1 interaction with PD-L2 on B cells. As mentioned before, the use of PTCy is increasingly applied to prevent cGVHD and a recent study in murine cGVHD suggests a critical role for Treg-B cell interactions in its effect. In this model PTCy, but not other prophylactic regimens inhibited initial alloreactive T cell responses, which led to increased Tregs and restored bone marrow B cell lymphogenesis (111).

Additional pathologic functions of B cells such as production of a large number of proinflammatory cytokines including IL-2, tumor necrosis factor (TNF)- α , IL-6, IL-12, and interferon- γ that activate immune cells such as T cells, macrophages, and natural killer (NK) cells have been shown to be involved in cGVHD pathogenesis (1, 8). Recently, Lv et al. observed that amongst other cells also activated B cells can produce IL-39, which might promote the expression of IL-39 receptors on CD4⁺ T cells (38). Overexpression of IL-39 promoted the aggravation of cGVHD development in two

different murine models, suggesting a critical role of IL-39 in the pathogenesis of cGVHD (see previous chapter).

In conclusion, B cell subpopulations and their associated signaling pathways as well as coordinated T cell and B cell responses have a key role in the pathogenesis of cGVHD. Pharmaceutical attempts to restore B cell homeostasis and target rare, pathogenic B cell subpopulations offer a new opportunity to reverse dysregulation of adaptive immunity in cGVHD.

B cell-derived EVs regulate T cell activation

When stimulated with CD24 or IgM, B cells are able to transfer functional BCR to recipient B cells *via* EVs (112). This was demonstrated by Phan et al. in a murine knockout model, where donor cells expressing GFP were co cultured after stimulation with IgM and CD24 knockout cells, resulting in the transfer of lipids and functional BCR to recipient cells, allowing for recognition of antigen and response (112). EVs from 'healthy' B cells are an area of limited research, however, there are interesting findings from B cell malignancies. Chronic lymphoid leukemia (CLL) B cells stimulated with CD40 and IL-4 release EVs containing miR-363, which can be transferred to CD4⁺ T cells and has a repressive effect on expression of CD69, a marker of activation in T cells (113). In this way, not only can more prominent immunosuppressive cell types such as Tregs or DCs subdue effector cells, but B cells can also repress CD4⁺ T cells. Co-stimulatory CD40L from EVs has also been demonstrated to have an effect on B cell response (114), whereby CD4⁺ T cell derived EVs that contain CD40L in their cargo, rather than on their surface, aid in the efficacy of HBsAg vaccination, increasing B cell activation, proliferation, and antibody production without impacting the surface expression of CD40 or CD80 on B cells (115). While this type of response is beneficial in the context of vaccination, increased aberrant B cells and concomitant antibody production could lead to aggravation of cGVHD. EV transfer of microRNAs from T cells to B cells also affect the immune response. Indeed, EV transfer of miR-25-3p and miR-155-5p, alongside other miRNAs such as miR-20a-5p, resulted in the silencing of the phosphatase and tensin homologue (PTEN) and Bcl-2-interacting mediator of cell death (BIM) genes. Both are known to have a critical function in B cell biology and the germinal center reaction, suggesting their role in germinal center formation and antibody production (116). Therefore, future research is needed to determine if aberrant EV signaling from B cells are amplifying disease mechanisms in the context of cGVHD.

Substantial dysregulation in the B cell compartment, in subset composition, pro-inflammatory signaling and disturbed T cell crosstalk is a specific feature of cGVHD and not aGVHD and therefore is critically important to understand in order to prevent and effectively treat this specific disease manifestation after HSCT. Restoring T cell function impacts the B cell compartment and vice versa, however, multiple factors determine these interactions and although there are many promising therapies recently approved or in the pipeline, it is still hard to predict which patients respond best

to which treatment. Investing research in robust biomarkers for both B and T cell dysregulation as the two major adaptive immune arms driving pathology will help to stratify patients and improve treatment plans.

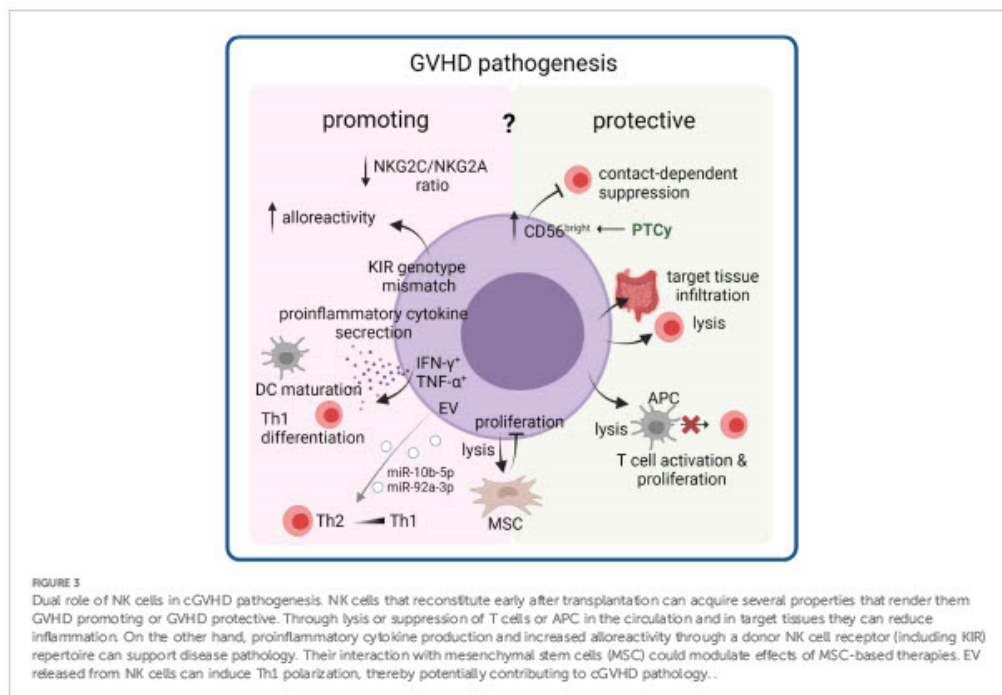
NK cells communicating within the cGVHD microenvironment

Natural killer (NK) cells are the first reconstituted immune cells to appear after allo-HSCT (117). Additionally, donor-derived NK cell infusion is increasingly studied as a prophylactic measure after allo-HSCT to target residual cancer cells (118, 119). However, the role of NK cells in cGVHD is not clear (120), and may depend on various factors and interactions within the microenvironment (Figure 3).

Activating and inhibitory NK cell receptors display dynamic expression patterns after HSCT and in cGVHD

Specific activity of NK cells is fine-tuned by expression of both activating and inhibitory receptors, which form a system of dynamic equilibrium that regulates NK cell responses (121). Among the various receptors present on NK cells, killer immunoglobulin-like receptors (KIR) (122) are a family of highly polymorphic receptors that includes members with both activating and inhibitory properties (123, 124). KIRs mostly recognize HLA class I molecules as their ligands (125, 126). KIR genotype mismatching was shown to increase cGVHD in HSCT patients undergoing myeloablative conditioning (127). Furthermore, KIR licensing status was significantly associated with cGVHD incidence in a univariate analysis (128).

Aside from KIRs, there is a wide variety of other NK cell receptors of both activating and inhibitory function. While not as polymorphic as KIRs, the genes coding for these receptors and their ligands harbor various important single nucleotide polymorphisms (SNPs) that can affect their function or expression (129), and may be associated with GVHD, including its chronic form (130). The natural cytotoxicity receptor (NCR) family includes three activating NK cell receptors: NKP46 (NCR1), NKP44 (NCR2), and NKP30 (NCR3) (131–133) with ligands that are not well defined (134–138). Another important NK cell receptor is DNAX Accessory Molecule-1 (DNAM-1), also known as CD226 (139). Its ligands are the adhesion proteins CD112 and CD155 (140). The CD92/NKG2 family includes both activating (e.g. NKG2C) and inhibitory receptors (e.g. NKG2A), encoded by genes located on chromosome 12 (141). The activating/inhibitory pair NKG2C/NKG2A is particularly important in NK cell maturation after HSCT. Interestingly, both receptors were shown to be downregulated in patients with extensive cGVHD (as compared to patients with no or mild cGVHD). However, the NKG2C/NKG2A ratio was decreased, suggesting a less activating profile of NK cells in patients developing extensive cGVHD (142).



NK cells communicate with other cGVHD cellular players

NK cells use their receptors and cytokine release to interact with other cells. They can stimulate maturation of APCs such as dendritic cells (DCs) by releasing TNF- α and IFN- γ and can also kill immature DCs *via* NKP30 and DNAM-1 receptors (143–145). Cytokine secretion by DCs themselves can promote cytotoxicity and cytokine production of NK cells (146). In mice, IFN- γ secretion by NK cells was able to promote differentiation from naïve CD4⁺ T cells to Th1 cells, which are important in cGVHD development (147–149). In both mice and humans, activated NK cells can specifically kill activated and proliferating CD4⁺ and CD8⁺ T cells in a perforin-dependent manner. This process is mediated by NK cell receptors NKG2D and DNAM-1, whose expression may be upregulated on activated T cells (150–152). NK cells can also suppress CD8⁺ T cells by releasing IL-10 (153, 154).

Mesenchymal stem cells (MSCs) are another cellular subpopulation known to interact with NK cells. A study on IL-2-activated NK cells showed that they can lyse MSCs, and this action likely involved NKP30, NKG2D, and DNAM-1 receptors. At the same time, MSCs inhibited proliferation of resting NK cells (155). The decreased NK cell proliferation corresponded with increased cytokine secretion and cytotoxicity, although some studies found these NK cell functions to be decreased instead (156–159). NK cell interactions with MSCs are of particular interest, due to the development of MSC-based treatment for cGVHD (160, 161). NK

cells may also target APCs (162), which are important drivers of cGVHD, as described in the following chapter. This APC-killing effect is potentiated in the presence of activating KIR2DS1 receptor binding to HLA-C2 on APC surface (163). Another receptor playing a role in this interaction may be NKP46, which binds to ligands present on DCs, and absence of NKP46 on NK cells leads to significantly increased GVHD (164, 165). NK cell degranulation towards activated T cells was decreased when either NKG2D, NKP46, or DNAM-1 was blocked, and decreased degranulation was associated with increased risk for cGVHD and aGVHD (166). However, the beneficial role of cytotoxic NK cell activity may depend on the disease stage, as patients with late aGVHD and cGVHD have increased numbers CD56dim cytolytic NK cells (75). Whereas NK cell cytotoxic activity can protect against GVHD development, it was shown that NK cells producing pro-inflammatory cytokines can induce it. A GVHD-stimulating effect of IFN- γ and TNF- α production by NK cells contributed to GVHD in both mice and humans (167, 168).

NK cell reconstitution dynamics impact cGVHD development

Due to their rapid reconstitution after HSCT (117), NK cell count reaches its normal numbers after one month as compared to T cells, whose numbers are usually still low three months post-transplant (117, 169). However, the circulating NK cells as late as

three months post-transplant are mostly of the CD56^{bright} subset, constituting nearly 40% of all NK cells in the transplanted patient, compared to only 10–15% in healthy individuals (whose NK cells are mostly of the CD56^{dim} subset) (170). CD56^{bright} NK cells are characterized by high expression of the inhibitory receptor NKG2A. CD56^{bright} cells in post-transplant patients are also reported to have unusually impaired IFN- γ secretion (169, 171).

Presence of T cells in the graft seems to have a positive effect on reconstituted NK cells and their development, as it increases their cytokine production and cytotoxicity (167, 172). However, both cellular subsets seem to have a different effect on cGVHD development, as e.g. donor-derived naïve T cells can increase the risk of cGVHD (47). While T cells can exacerbate GVHD, NK cells are able to suppress it. Low reconstitution levels of NK cells were linked to a poorer outcome in cGVHD patients (173) and low numbers of reconstituting NK cells as well as a dysfunctional phenotype is associated with aGVHD relapse in patients receiving PTCy (174). One of the possible mechanisms behind this is lysis of alloreactive T cells, mediated by NK cell receptor NKG2D. This process leads to killing by induction of FasL-mediated apoptosis and degranulation. This occurs possibly due to increased expression of an NKG2D ligand on alloreactive T cells during aGVHD, as well as trafficking of NK cells to GVHD-affected tissues driven by chemokine release (175, 176). However, this mechanism has not been verified specifically in cGVHD patients as of yet. Interestingly, a study on mice showed that even residual recipient NK cells could prevent aGVHD by targeting T cells in a minor antigen mismatch setting (177). The CD56^{bright} NK cells were observed to be decreased in patients developing cGVHD (46, 178–180). Lauener et al. demonstrated that HSCT patients later developing cGVHD already displayed with lower levels of CD56^{bright} cells at day 100 compared to GVHD-free HSCT patients (181), suggesting a causal involvement of this subset in cGVHD. However, it is so far unclear what causes the loss of CD56^{bright} cells in patients prior to cGVHD and how this could be prevented. Interestingly, patients receiving PTCy have delayed T and NK cell reconstitution with a higher proportion of immature CD56^{bright} CD16[−] NK cells compared to patients receiving conventional GVHD prophylaxis (174, 182). In a cohort of haplo-HSCT transplanted an increased ratio of CD56^{bright}CD16[−] immature NK cells to CD56^{bright}CD16⁺ mature NK cells was correlated with the development of cGVHD (182), suggesting that the protective effect of the CD56^{bright} population depends on the maturation status. A higher proportion of CD56^{bright} cells is also partly responsible for the therapeutic effects of extracorporeal photopheresis (ECP) treatment in cGVHD (183). The protective effect of CD56^{bright} NK cells may be due to their ability to suppress effector T cells in a contact-dependent manner (184). This may be mediated by NCR receptors NKp44 and NKp46, although not all studies support this (181, 184). This regulatory NK cell subset was also described to be perforin⁺, Granzyme B⁺ and NKp46⁺ (181).

NK-cell derived EVs can polarize T cells

Activated NK cell-derived EVs (NK-EV) also have potential for a direct effect on cells, whereby EVs containing cytotoxic molecules

such as perforin or FasL (185) have been shown to exhibit antitumoral effects. However, NK-EV miRNA have also been demonstrated to play a functional role in the polarization of a CD4⁺ T cell response. EVs containing miR-10b-5p and miR-92a-3p were shown to downregulate GATA3 mRNA, the Th2 signature transcription factor; resulting in an increase of Th1-like cells producing Interferon gamma IFN- γ , and Interleukin-2 *in vitro* (186). *In vivo* this would exacerbate or sustain the pro-inflammatory shift seen in cGVHD.

The dual role of NK cells *via* cytotoxicity and production of pro-inflammatory cytokines, as well as complicated interactions between T cells and reconstituted NK cells, may be responsible for discrepant results of studies on NK cell in human GVHD development. It is hypothesized that significant pre-activation of NK cells can lead to their GVHD-inducing character to prevail over their T cell killing capabilities (120). Although the role of NK cells in GVHD is not entirely clear, many studies have reported that early reconstitution of NK cells is associated with favorable outcomes, suggesting that by maximizing NK cell functions, HSCT outcomes could be improved (187).

Antigen presenting cells as disease drivers in cGVHD

Professional APCs are a heterogeneous group of immune cells, that are highly efficient in processing and presenting antigens to promote an adaptive immune response. Major populations of APCs in human tissues include DCs, macrophages and - to a lesser extent - cells of the B-lineage. Together with co-stimulatory signals upon formation of an immunological synapse, APC may activate CD4⁺ T cells by exogenous antigen presented *via* MHC-II, and cytotoxic T cells *via* presentation of endogenous antigen on MHC-I (Figure 4).

Host and donor APC can initiate pathologic T cell responses in acute and chronic GVHD

In the context of HSCT, host (tissue) antigens can be presented by host APCs both *via* MHC-I and MHC-II or, indirectly, cross-presented by donor APCs *via* MHC-I (188–191). With this in mind, several studies around the turn of the millennium have focused on investigating the presence of resident host APCs that survive conditioning and have the ability to initiate GVHD. Interestingly, during pre-transplant conditioning, the APC compartment of barrier tissues remains fairly unaffected, and replacement with donor APCs after HSCT is incomplete (192–194). Together with the presence of remaining recipient tissue-resident T cells (51, 52), this results in mixed chimerism of both APC and lymphocyte populations within tissues after HSCT. Indeed, due to their capacity to present host allo-antigens, host APCs are required for initiation of GVHD responses in tissues (195, 196). However, the majority of APCs in GVHD lesions were found to be of donor

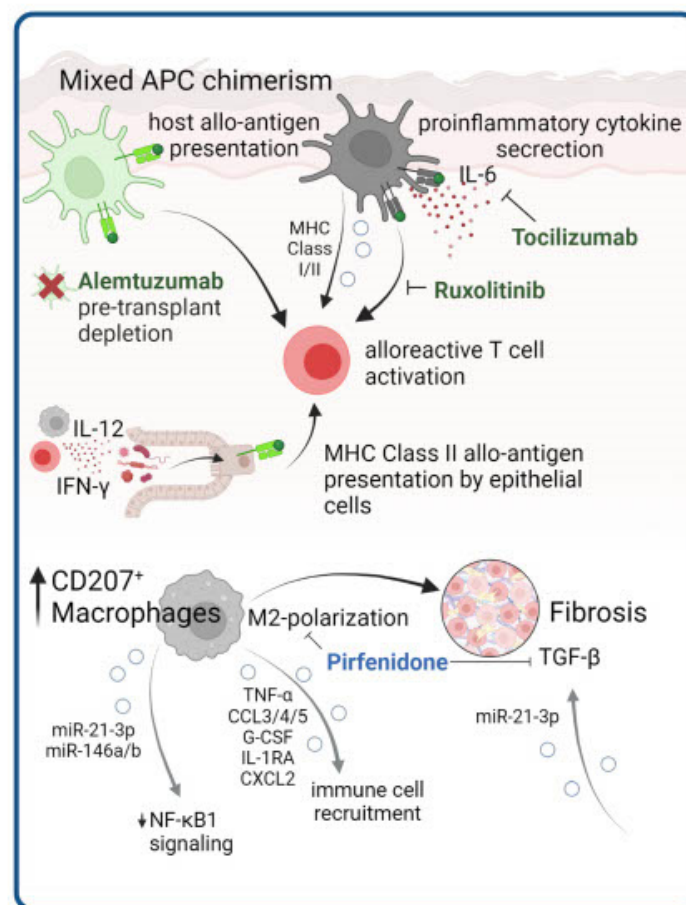


FIGURE 4

APCs as cGVHD disease drivers. APCs are instrumental in cGVHD development through presentation of allo-antigens to T cells and production of pro-inflammatory cytokines. Antibodies targeting these mechanisms are in clinical use (in green). Structural cells, such as epithelial cells can obtain MHC class II antigen-presenting properties presenting allo-antigen in the gut of mice. In cGVHD macrophages with M2 polarization are expanded and contribute to tissue fibrosis, which in mouse models could be ameliorated by pirfenidone. There are several lines of evidence that EV release by APC mediate processes implicated in cGVHD pathology.

origin (197, 198), suggesting a prominent role for host APCs in the propagation of inflammation by cytotoxic T cell activation.

In consequence, preventive and therapeutic strategies for GVHD have focused both on depletion of host APCs, for example by pre-transplant conditioning regimens containing alemtuzumab (CAMPATH) (199), and the modulation of donor APC cytokine responses, including IL-6-blockade with tocilizumab (200). We have above discussed the effects of Ruxolitinib on pathogenic T cell signaling, however this drug also can modulate APC function. Ruxolitinib impairs human DC activation and DC-mediated T cell activation (201), thereby additionally benefiting therapeutic effects.

Regarding specific APC types in GVHD inflammation, there is a redundancy as to which cells can initiate GVHD. In skin, host Langerhans cells are potent activators of donor cytotoxic T cells (202) and cutaneous aGVHD results in replacement by donor LCs, suggesting activation and migration (194). However, LC are not essential to murine GVHD and in the absence of LCs, other APC subsets may drive inflammation (203).

In murine models of cutaneous and pulmonary cGVHD the infiltration of M2-polarized donor F4/80⁺ macrophages preceded disease development and blocking this infiltration with anti-CSF-1R antibody significantly reduced cGVHD in both organs (198). Expansion of myeloid cells expressing the type-2 macrophage

marker CD163 in steroid refractory aGVHD (204), and CD207⁺ donor-derived macrophages in cGVHD-affected tissues (198), suggest overall M2-polarization of GVHD macrophages. Although macrophage polarization profiles have not been formally investigated in GVHD subtypes in humans, the high plasticity of macrophages to exert both pro-inflammatory and pro-fibrotic functions could indicate a role in non-classical GVHD subtypes. Of note, Adams et al. recently demonstrated that BM-derived MHC class II⁺ macrophages are critical mediators of neuroinflammation in a cGVHD mouse model (205). During murine skin aGVHD, a distinct donor-derived macrophage population expands, and maintains a proinflammatory phenotype even after GVHD resolution, leading to a loss of tolerance and Treg suppressive capacity, which might further prime chronic inflammation and fibrosis (206). Du et al. treated mice with cGVHD with the antifibrotic small molecule Pirfenidone, which was FDA-approved for treating idiopathic pulmonary fibrosis. This resulted in reduced donor BM-derived M2 macrophage infiltration, decreased expression of pro-fibrotic TGF- β , and reduced lung fibrosis (207).

Compared to macrophages, DCs show more rapid engraftment and increased donor chimerism after HSCT (208). Common DC subsets in human tissues include conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (209). While cDC were shown to be a critical donor APC presenting allo-antigen after HSCT (210), recovery of donor pDCs early after HSCT reduces GVHD (211, 212).

Antigen presentation by non-professional APC contributes to inflammation

Finally, more recently, non-hematopoietic cells have come into focus as cells that can develop antigen-presenting ability under specific conditions. This includes keratinocytes (213), fibroblasts (214), alveolar epithelial cells and intestinal epithelial cells, the latter of which were found to initiate lethal gastrointestinal GVHD by MHC class II-presentation of allo-antigens (215). Koyama et al. demonstrate in a mouse model of GI-GVHD that IL-12 from ileal macrophages, IFN- γ from lymphocytes, as well as sensing of microbiota-derived TLR ligands induces MHC-II upregulation on intestinal epithelial cells, which then present allo-antigens to donor T cells to induce lethal GVHD (215). This study already highlights the complex interactions with the microbiome in GVHD target organs at barrier sites, which will be discussed in the next chapter.

Apart from antigen presentation, non-hematopoietic cells, specifically fibroblasts are involved in cGVHD pathogenesis as main executors of tissue fibrosis by excessive production of extracellular matrix and collagen deposition in response to chronic inflammation. Interestingly, a recent report demonstrates that fibrosis could be amplified through EV signaling. Dermal fibroblasts that were exposed to cGVHD patient derived EVs displayed significantly higher levels of fibrosis mediator TGF- β compared to healthy EV exposure (216). Another preliminary study in a small cohort of cGVHD patients found significantly

increased miRNA miR-29-3p in patient EVs, which was predicted to regulate hub genes involved in TGF- β signaling and fibrotic processes, such as type I collagens or MMP2 (217).

EVs regulate antigen presentation

EVs also play a key role in antigen presentation, whereby EVs released by APCs carry surface MHC Class-I and MHC Class-II molecules and can directly stimulate CD8⁺ and CD4⁺ T cells, respectively (218, 219). The capacity of EVs to stimulate T cells, including naïve T cells, can be enhanced by their interaction with DCs. The presence of specific adhesion molecules that are involved in EV binding to DCs, such as integrins and ICAM1, depends on the lineage and activation stage of the parent cells (220). Indeed, EVs released by LPS-treated DCs bear more MHC Class-II and exhibit a more potent T-cell stimulatory function than EVs secreted by immature DCs. Indeed, mature DC EVs transfer the ability to activate naïve T cells to non-professional APCs (221, 222). A percentage of the acquired EVs remain on the surface of the target cell, whereas the rest are internalized by phagocytosis or micropinocytosis. Immature DCs internalize EVs more efficiently than mature DCs, whereas mature DCs retain more EVs on the cell surface (223). Once internalized by DCs, the EV-derived peptide-MHC (p-MHC) complexes can be degraded by the APCs and used as a source of peptide to interact indirectly with T-cells. p-MHC complexes of EVs attached (or fused) to APC surfaces can also be presented directly to T-cells without the need for p-MHC reprocessing, through a mechanism known as cross-presentation (222). The finding that optimal T-cell stimulation occurs when EVs transfer p-MHC complexes to mature DCs indicates that the acceptor APCs provide the required costimulatory molecules that are absent in the EVs (224). There are several lines of evidence suggesting that, following allograft transplantation, cross-presentation of recipient's APCs with donor MHC molecules could be mediated through EV transfer (225).

Professional and non-professional antigen presenting cells were shown to provide either pro- or anti-inflammatory functions in GVHD, depending on the specific cell type and context. Notably, APC can drive GVHD using different mechanisms including direct priming of T cells, release of EV and contribution to the cytokine milieu. As such, APC are promising targets in therapeutic modulation of T cell responses in GVHD.

Microbiome-mediated effects in cGVHD

The gut microbiome is defined as the genetic and functional profile of the microbes residing in the gut. Together, these microbes encode over three million genes, providing a much wider variety and flexibility than the 23,000 protein-coding genes of the human genome (226, 227). The microbiota have gained increasing attention for its influence on human health and disease, and has

been shown to influence the epithelial mucus layer, promote development of lymphoid structures, function in activation and differentiation of lymphocyte populations and balance the production of IgA and antimicrobial peptides (228) (Figure 5).

Mycobiome dysbiosis occurs after allo-HSCT

The microbiome comprises bacteria, fungi and viruses, however in the context of GVHD studies mostly focus on the bacterial microbiome. We are only beginning to understand the role of the mycobiome in aGVHD. In a retrospective study of 153 allo-HSCT recipients, patients with intestinal *Candida* spp colonization showed significantly more grade II-IV aGVHD (229). Rolling et al. profiled mycobiota dynamics in allo-HSCT patients and found stable overall fungal diversity, but high day to day variance. Interestingly, stable expansion of *Candida parapsilosis* complex species in fungal cultures associated with higher transplant related mortality (230). It remains to be determined if there is causal role for fungi in the development or promotion of aGVHD and cGVHD.

Gut microbiota composition influences GVHD development

The bacterial microbiome has emerged as a key player in GVHD development and progression. It has long been postulated that germ-free mice have prolonged survival compared to conventional mice after allo-BMT (231, 232). However, this was recently challenged by a study showing that after allo-BMT, germ free mice displayed greater GVHD than age-matched littermates kept in specific pathogen free conditions, indicating that the microbiome itself can mitigate GVHD (233). There is a plethora of studies demonstrating that the gut microbiota, through their metabolites, are able to influence host immune responses, and that loss or gain of certain bacterial taxa may influence GVHD pathology. The importance for microbiota on GVHD development is covered in detail in recent excellent reviews (234, 235). The composition of the microbiome at the time of transplant or post-transplant can impact the outcome and risk for developing aGVHD and cGVHD. Numerous studies have shown that the severity of GVHD after allo-HSCT is associated with decreased microbial diversity of the GI tract, with a particular loss of

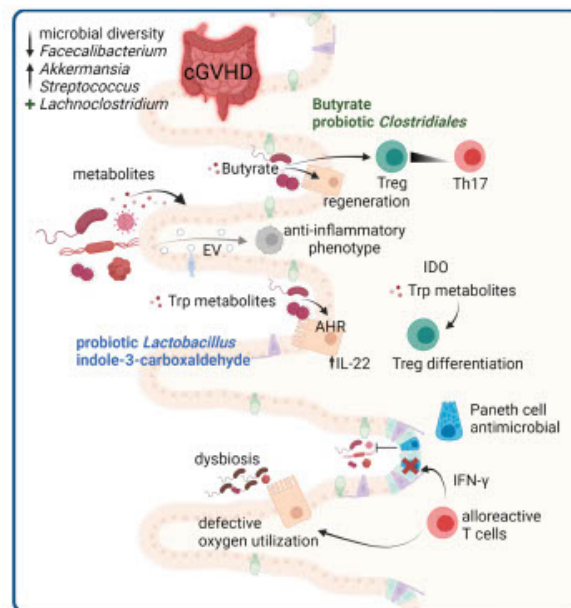


FIGURE 5

Microbiome-mediated effects in cGVHD. GVHD development in the gut is associated with a disturbed microbiome. In cGVHD decreased microbial diversity with reduced *Faecalibacterium* and increased *Akkermansia* and *Streptococcus* species are reported, whereas high abundance of *Lachnospirillum* is protective. Microbial metabolites are critical mediators of gut immune responses. Butyrate promotes Treg and regeneration of epithelial cells. Trp metabolites derived from microbes can protect the epithelium via IL-22 induction and microbial or tissue cell derived Trp metabolites promote Treg differentiation. Paneth cells in the gut produce antimicrobial peptides, and are targets of destruction by alloreactive T cells, leading to impaired host defense. Damaged epithelial cells have defective oxygen utilization, leading to loss of physiologic hypoxia in GVHD which drives microbial dysbiosis. Microbiota can release EV that modulate host immune responses.

commensal anaerobes (236–238). Seike et al. recently discovered that dysbiosis in the gut of allo-BMT mice is a consequence of a defective oxygen utilization by intestinal epithelial cells, caused by pathogenic T cell attack, leading to a loss of physiologic hypoxia (233). In this model, dysbiosis before transplantation did not affect GI-GVHD severity, arguing that dysbiosis is more a consequence than a cause for GI-GVHD. In a case-control study with collected stool samples, Markey et al. showed that a reduction in *Faecalibacterium* and an increase in *Akkermansia* and *Streptococcus* was associated with development of cGVHD (239). In the same study, increased abundance of *Lachnospiraceae* and *Clostridium* at day +100 after transplantation was associated with better prognosis and cGVHD-free survival (239). For aGVHD, several studies have indicated that increased abundance of *Lachnospiraceae* is associated to reduced risk and increased overall survival of aGVHD (238, 240, 241). It is not well understood yet how GVHD prophylaxis with PTCy affects the microbiome, however cyclophosphamide-microbiome interactions have been studied in the context of cancer. In antibiotic treated mice the anti-cancer activity of cyclophosphamide was reduced, suggesting a crucial involvement of the microbiome in the immunomodulatory activity of the drug (242). Dalli  re et al. demonstrated that in mice *Enterococcus hirae* translocate to secondary lymphoid organs during cyclophosphamide treatment, leading to an increase in intratumoral CD8⁺Treg ratios, while *Barnesiella intestihominis* facilitates the infiltration of IFN-   producing    T cells (243). How dysbiosis in HSCT patients is affected by PTCy and whether there are any implications for host immune responses and GVHD pathobiology must be addressed in future studies.

Microbial metabolites affect the epithelium and host immunity

The commensal bacteria influence host immunity through direct interactions with the epithelium, or *via* metabolites. Short chain fatty acids produced by anaerobic bacterial fermentation of dietary fibers in the colon have been implicated in mediating epithelial layer integrity and optimal immune cell homeostasis in the gut. Of these, butyrate produced by Firmicutes such as *Clostridia*, modulate immune cell phenotypes and composition, and in particular the skewing towards Tregs at the expense of Th17 (244). This has important implications, as an over-weight of Th17 cells can drive chronic GI GVHD due to production of profibrotic cytokines (245). Butyrate also contributes to epithelial cell regeneration. A study by Mathewson et al. demonstrated that administration of butyrate or a probiotic cocktail of 17 Clostridiales species to allo-BMT mice led to amelioration of GVHD through improved epithelial layer integrity (246). Similarly, administration of selective antibiotics that preserve the presence of butyrate-producing bacteria lead to reduced GVHD mortality in allo-HSCT patients (247). In line with these finding, a lower plasma concentration of butyrate and propionate on day 100 was observed in patients that developed cGVHD (239). However, there is conflicting data as another study has shown abundance of

butyrate-producing bacteria in patients developing steroid-refractory cGVHD (248).

Among other metabolites, the essential amino acid tryptophan is metabolized to kynurenin by the enzyme indoleamine 2,3-dioxygenase (IDO), expressed in intestinal epithelial cells and immune cells. A significant proportion of tryptophan is also metabolized to tryptamine and indole metabolites through tryptophanase and decarboxylase enzymes, *via* the *Lactobacillus* and *Clostridia* genus (249). Tryptophan metabolites interact with the nuclear transcription factor aryl hydrocarbon receptor, which leads to increased production of the epithelial protective cytokine IL-22 by ILC3s (250). Probiotic administration of *Lactobacillus* was shown to reduce GVHD in a mouse model (236), and another mouse study showed that oral administration of the indole metabolite indole-3-carboxaldehyde reduced gut epithelial damage and reduced overall GVHD pathology and mortality (251). The mechanism was demonstrated to involve *Lactobacillus* and ILC3s (252). In addition, tryptophan metabolism through IDO also promotes Treg differentiation (253), and has been associated with suppression of GVHD in animal models (254).

Maintenance of an optimal bacterial diversity is governed by multiple external and intrinsic factors. Within intestinal crypts, specialized cells with antimicrobial functions, the Paneth cells, play a role in maintaining the homeostatic balance between host and the microbiome. Upon pre-conditioning prior to allo-HSCT, the epithelium may be damaged, and loss of crypt structures has been associated to delayed recovery from GVHD and steroid-refractory GVHD (255–257). The disruption of crypts is accompanied by fewer Paneth cells, that produce essential anti-microbial peptides and growth factors necessary for maintenance of intestinal stem cells. Crypt destruction was recently demonstrated to be mediated in an IFN-  -dependent mechanism by alloreactive T cells that invade the crypt structures early after transplantation (258, 259).

Microbiota-derived EVs regulate host immunity

The immune modulating effects of EVs are not constrained to self-cells. Microbiota have also been found to excrete EVs, which are more akin to shedding microvesicles. These outer-inner membrane vesicles (O-IMVs) have been described by P  rez-Cruz et al., which in their study derived from several pathogenic Gram-negative bacteria and contain cytoplasmic components (260). Like EVs, these particles display and encompass host cell proteins and nucleic acids, which are known to elicit an immune response (260). Contrarily, EVs produced by beneficial microbes such as *Clostridium butyricum* polarize macrophages into an anti-inflammatory phenotype, aiding in treatment and remission of a murine colitis model (261). Though the potential exists for anti-inflammatory O-IMVs, the dysregulation of the various microbiomes as a result of cGVHD may shift towards pathogenic microbes and exacerbate disease. Further research is necessary to investigate these effects in pre-clinical models and patients.

The described studies highlight the potential of manipulating the bacterial composition or metabolites to not only promote

epithelial regeneration, but to also promote an immune cell composition that may ameliorate and prevent the damage caused by alloreactive donor T cells, thus indicating that manipulation of bacterial metabolites can be exploited clinically. Therapeutic strategies in terms of cGVHD should involve prebiotics or probiotics that skew the balance in favor of bacterial strains that provide beneficial metabolites and out-compete strains with an unfavorable impact. Alternatively, the use of fecal transplantations, that has been shown to ameliorate GVHD in patients with steroid-refractory aGVHD (262), may be considered.

Discussion

Understanding cellular networks within cGVHD-affected tissue will pave the way forward to apply precision medicine

In this review we summarized current knowledge about disease mediating signaling in different cell types involved in the pathogenesis of cGVHD. Disease complexity and heterogeneity is reflected by the involvement of different cellular players described here, displaying multiple options for inter- and intracellular signaling that can either promote or inhibit cGVHD. While compartmentalization of the immune system into different cell types within specific organs is useful study single mechanisms, in disease all the described pathways can be active in the given tissue and organism at the same time. The difficulty lies in the deconvolution of these signaling networks, to find targets for effective preventive and therapeutic treatments.

A lot of our knowledge about cellular disease mechanisms comes from mouse studies. With the advance of (spatial) single-cell technologies we are gaining a better understanding of human tissue- and disease specific pathways driving certain phenotypes (263–266). However, there is high variability between patients, and at present markers to predict therapy response or disease outcome are lacking.

In the age of precision medicine, we require reliable biomarkers for patient stratification to determine the best possible treatment for every individual patient. The collection of large-scale open-source OMICs data from patient cohorts all over the globe is increasing, as is the development of sophisticated bioinformatic tools to predict cell-cell communication and tissue organization in homeostasis and inflammation (267–271). With the rapid improvement of deep learning methods, biologists and clinicians have the tools at hand to better understand cGVHD pathology from a systems perspective and uncover disease-associated processes that have high therapeutic potential and can be tailored to the patients' specific immune status (272).

As lately discussed in the NIH Consensus document on personalized treatment for cGVHD (273), it will be crucial to understand the connection between clinical effects and the underlying biological mechanisms in cGVHD therapies currently in use, which will also allow the development of new, refined therapeutic approaches. Importantly, the authors emphasize the need for conducting clinical trials for first line treatment without the

concomitant use of glucocorticoids, which hamper the investigation of disease-modulating effects of the used therapeutics in those trials (273). Close monitoring of patients during treatment with intensive sample collection and biological analysis will be necessary to improve our understanding of cGVHD pathogenesis, and definition of reliable biomarkers which can be used to design personalized treatment schemes (273). Ultimately, a better understanding of immune cellular crosstalk and its regulation in cGVHD will lead to the development of better drugs and therapies and improve the life of patients.

Studying cellular crosstalk via EVs is a new way forward in cGVHD diagnosis, patient stratification and development of therapeutics

Collectively, the studies highlighted in this review demonstrate that the activities of EVs can affect wide ranging immunoregulatory functions on a variety of immune cells which are known to be involved in the etiology of GVHD, including modulating antigen presentation, immune activation, immune suppression, immune surveillance, and intercellular communication, *via* both direct and indirect mechanisms. Given the ubiquitous involvement of EVs in immunomodulation, their translational potential as biologically relevant biomarkers and as novel therapeutics offers an exciting area for future discovery in the GVHD field. Despite this however, many questions remain unanswered and will be the focus of immediate research, particularly to decipher their precise roles in post-HSCT immune cell crosstalk and regulation. As a field in its relative infancy, fundamental questions remain about the best way to isolate, characterize and study EVs, including the effect of variation in sample collection, storage protocols and cell manipulation on EV productivity and molecular cargo. This in turn influences comparability between GVHD studies, which are already made challenging due to the heterogeneity in sample cohorts in terms of HSCT conditioning, prophylaxis and GVHD therapies. Many of these questions around best standardization in EV research are the topic of intense scrutiny by the International Society of Extracellular Vesicle (ISEV) specialist task force groups. Moving towards future EV research, there will be much focus on the heterogeneity of EV populations themselves, including those produced by single immune cell types, as well as the variation in EV populations in the circulation and how these can be traced back to immune cell of origin. Given the key advancements in our understanding of the role of EVs in immune cell crosstalk, as well as the complexity of the immune system, immune cell derived EVs look to be an area of intense research and development in future years.

Author contributions

GS and LMG conceptualized the manuscript. LMG, KJS, PL, JS, SJB, ESU, KB-K, HTG, REC, MI, and GS wrote the manuscript.

LMG drafted figures and edited the manuscript. All authors contributed to the article and approved the submitted version.

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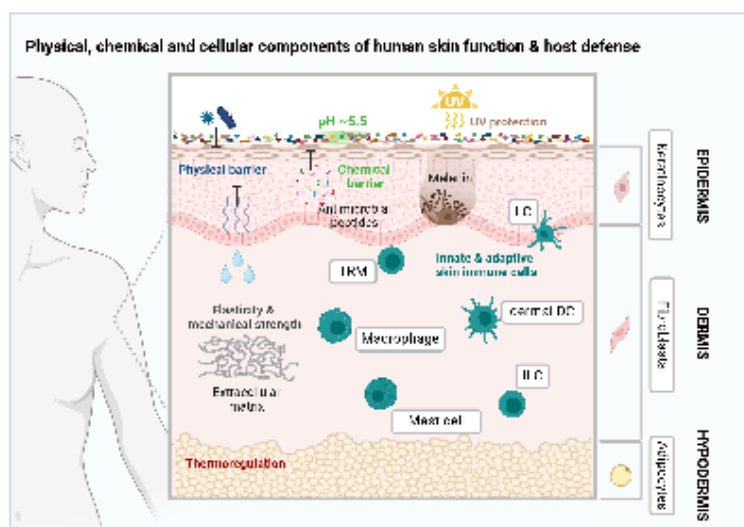
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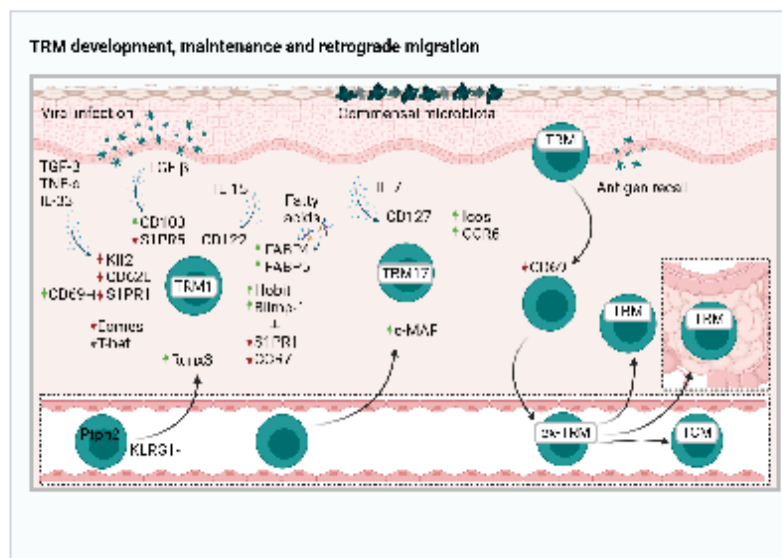
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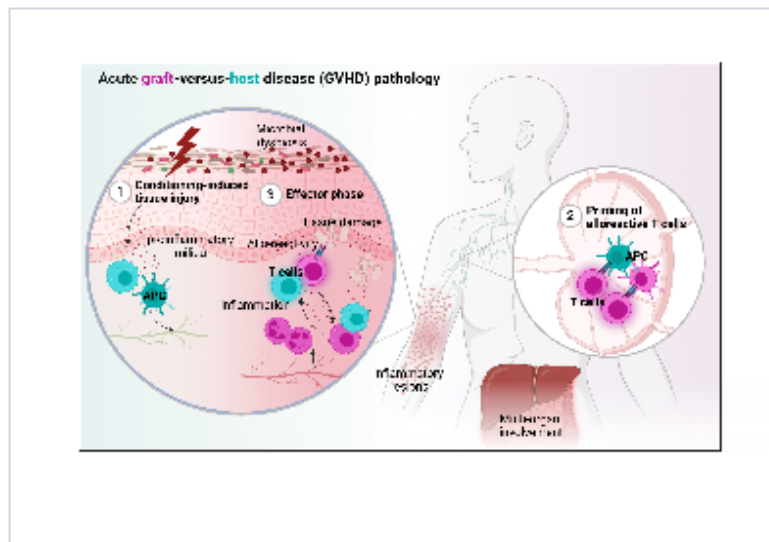
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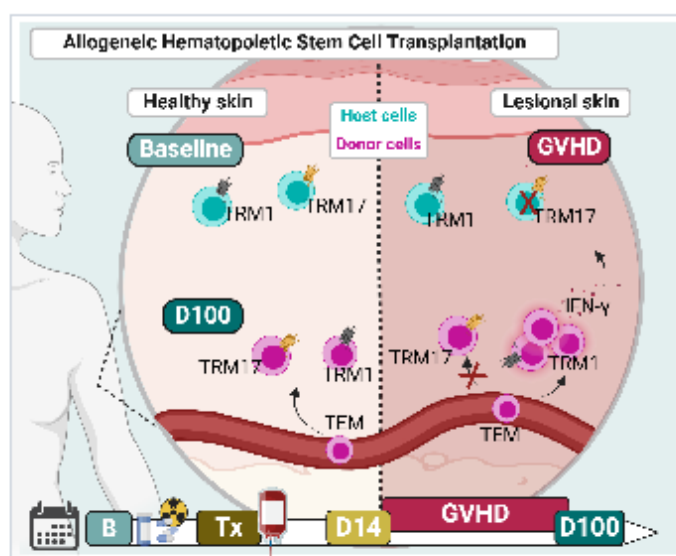
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CV

Curriculum vitae

LAURA MARIE GAIL



lauramarie.gail@gmail.com

EDUCATION

- September 2019 – present** **Doctoral studies, CeMM PhD program and PhD program 'Immunology' at the Medical university of Vienna**
Doctoral thesis: 'Regulation of skin tissue resident memory T cells in hematopoietic stem cell transplantation and acute graft-versus-host disease' under supervision of Univ.-Prof. Dr. Georg Stary
- October 2016 – March 2019** **Master of Science in Medical Biology, Paris-Lodron University, Salzburg**
Master thesis: 'Characterization and phenotyping of human immune cells in a humanized skin model in NOD-Scid-γ mice' under supervision of Assoc.-Prof. Iris Gratz, PhD
- October 2013 – July 2016** **Bachelor of Science in Biology, Paris-Lodron University, Salzburg**
Bachelor thesis: 'Inflammatory signaling in the brain in the context of depression' under supervision of Univ.-Prof. Josef Thalhammer, PhD
Specialization on Molecular Biology & Genetics
- September 2009 – July 2012** **Highschool, Holbein Gymnasium, Augsburg (Germany)**
Graduation subjects: German, English, Mathematics, Biology and Geography

RESEARCH EXPERIENCE

- November 2017 – February 2019** **Project staff, Iris Gratz lab, Paris-Lodron University, Salzburg**
Project: A humanized mouse model to study skin immunology
Relevant methods: human primary cell culture, mouse handling, injections, surgery & organ harvest, flow cytometry
- August 2018** **LISA Summer Academy, Hanover Biomedical Research School, Hanover**
Topic: Immunology, Inflammation and Regeneration – Basic concepts and novel therapeutic approaches
- July 2016** **Internship, IHC Routine lab, Institute for Pathology, University Hospital Salzburg**
Relevant methods: microtomy and paraffin embedding of primary human material

ADDITIONAL INFORMATION

FUNCTIONS

- January 2021 – present** **Next Generation Immunologists (NGI)**
Board member & secretary
- Young European Immunological Societies (yEFIS)**
Task force member & national representative of Austria (NGI)

AWARDS & FELLOWSHIPS

- June 2022** **Best Poster award YSA Symposium**
Young Scientist Association Symposium, Medical University of Vienna, June 14th – 15th 2022
- December 2022** **Wissenschaftspreis der ÖGDV**
Awarded by the Austrian Society of Dermatology and Venerology, with Dr. med. Univ. Johanna Strobl, for the publication:
Human resident memory T cells exit the skin and mediate systemic Th2-driven inflammation

January 2023	Keystone Symposia scholarship Keystone meeting Skin-Immune Crosstalk, Breckenridge, Colorado, USA
May 2024	EFIS-EJI Travel Grant for the ECI 2024 7 th European Congress of Immunology, Dublin, September 2024

CONFERENCE & MEETING PRESENTATIONS

2021	ÖGDV Science Days, 25th – 26th June 2021 Poster presentation
2022	YSA PhD Symposium, 14th – 15th June 2022 Poster presentation ADF Winter School, 23rd – 25th June 2022 Poster presentation ÖGDV Science Days, 15th – 17th September 2022 Oral presentation Austrotransplant annual meeting, 19th – 21st October 2022 Oral presentation
2023	Keystone Meeting Skin Immune crosstalk, 13th – 16th February 2023 Oral presentation ÖGDV Science Days, 15th – 17th June 2023 Oral presentation ÖGAI annual meeting, 14th – 16th September 2023 Poster presentation Inflammatory skin disease summit, 15th – 18th November 2023 Poster presentation
2024	Harald von Böhmer Midwinter Conference, 20th – 24th January 2024 Poster presentation European Congress of Immunology, 1st – 4th September 2024 Oral presentation

PUBLICATIONS

P Starkl, G Jonsson, T Artner, B Lenfers Turnes, **LM Gail**, T Oliveira, A Jain, N Serhan, K Stejskal, K Lakovits, A Hladik, M An, KM Channon, H Kim, T Köcher, W Weninger, G Stary, S Knapp, V Klang, N Gaudenzio, CJ Woolf, S Tikoo, R Jain, JM Penninger, SJF Cronin. Mast cell-derived BH4 and serotonin are critical mediators of postoperative pain. *Sci Immunol*, August 2024. doi: 10.1126/sciimmunol.adh0545

J Strobl, **LM Gail**, L Krecu, S Madad, L Kleissl, L Unterluggauer, A Redl, K Brazdilova, S Saluzzo, P Wohlfarth, HA Knaus, M Mitterbauer, W Rabitsch, M Haniffa, G Stary. Diverse macrophage populations contribute to distinct manifestations of human cutaneous graft-versus-host disease. *Br J Dermatol*. November 2023. doi: 10.1093/bjd/ljad402.

LM Gail, KJ Schell, P Lacina, J Strobl, SJ Bolton, E Steinbakk Ulriksen, K Bogunia-Kubik, H Greinix, RE Crossland, M Inngjerdigen, G Stry. Complex interactions of cellular players in chronic Graft-versus-Host Disease. *Front Immunol*. June 2023. doi: 10.3389/fimmu.2023.1199422

RV Pandey, J Strobl, A Redl, L Unterluggauer, **LM Gail**, L Kleissl, S Müller, D Atzmüller, V Fife-Gernedl, T Krausgruber, H Knaus, M Mitterbauer, P Wohlfarth, W Rabitsch, C Bock, G Stry. Epigenetic regulation of T cell lineage in skin and blood following hematopoietic stem cell transplantation. *Clin Immunol*, January 2023. doi: 10.1016/j.clim.2023.109245

N Bayer, R Vinay Pandey, B Hausman, F Deckert, **LM Gail**, J Strobl, P Pjevac, C Krall, L Unterluggauer, A Redl, V Bachmayr, L Kleissl, M Nehr, R Kirkegaard, A Makristathis, M Watzenböck, R Nica, C Staud, L Hammerl, P Wohlfarth, R Eckert, S Knapp, W Rabitsch, D Berry, G Stry. Disturbances in microbial skin recolonization and cutaneous immune response following allogeneic stem cell transfer. *Leukemia*. October 2022. doi: 10.1038/s41375-022-01712-z

S Saluzzo, **LM Gail**, T Neuwirth, R Vinay Pandey, M Farlik, G Stry. Processing human skin samples for single-cell assays. *STAR Protocols*, June 2022. doi: 10.1016/j.xpro.2022.101470

S Saluzzo, R Vinay Pandey, **LM Gail**, R Dingelmaier-Hovorka, L Kleissl, L Shaw, B Reininger, D Atzmüller, J Strobl, V Touzeau-Römer, A Beer, C Staud, A Rieger, M Farlik, W Weninger, G Stingl, G Stry. Irreversible damage of skin- and mucosa-resident memory T cells in patients with advanced HIV disease. *Immunity*, November 2021. doi: 10.1016/j.immuni.2021.10.021

J Strobl*, **LM Gail***, L Kleissl, R Vinay Pandey, V Smejkal, J Huber, V Puxkandl, L Unterluggauer, R Dingelmaier-Hovorka, D Atzmüller, T Krausgruber, C Bock, P Wohlfarth, W Rabitsch, G Stry. Human resident memory T cells exit the skin and mediate systemic Th2-driven inflammation. *J Exp Med*. October 2021, 218. doi: 10.1084/jem.20210417 (*shared first authorship)

MM Klicznik, A Benedetti, **LM Gail**, SR Varkhade, R Holly, M Laimer, A Stoecklinger, A Sri, R Reitsamer, T Neuper, J Horejs-Hoeck, MD Rosenblum, DJ Campbell, EM Muraier, IK Gratz. A novel humanized mouse model to study the function of human cutaneous memory T cells in vivo in human skin. *Sci Rep*. July 2020, doi: 10.1038/s41598-020-67430-7. (contribution during Masters thesis)