

Temporal immune niche dynamics during inflammation and hematopoietic stem cell transplantation

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Submitted by

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Declaration

I, Florian Deckert, declare that this doctoral thesis entitled “Temporal immune niche dynamics during inflammation and hematopoietic stem cell transplantation” was independently conceived and written by me. The thesis follows a cumulative format and is based on two manuscripts. At the time of thesis submission, one study (Gail and Deckert et al., 2026) is under revision at Nature immunology, and I contributed as co-first author. A second study has been submitted to Blood (Deckert et al., 2026), in which I am first author. In both works, I performed bioinformatic analysis and substantially contributed to the conceptual development of research questions, study design, interpretation of results and manuscript preparation.

Deckert F, Hladik A, Lakovits K, Farhat A, Pimenov L, Meng F, Fokina A, Quattrone F, Gorki AD, Gawish R, Knapp S

Myeloid IFN-I sensing boost splenic stress erythropoiesis
(submitted to Blood)

Gail LM*, Deckert F*, Thébault L, Dingelmaier-Hovorka R, Strobl J, Eskandari D, Heinzl L, Holzer E, Kleissl L, Reininger B, Mayerhofer C, Gabriel A, Freystätter C, Shaw L, Rüsing L, Cserna J, Weninger W, Farlik M, Rabitsch W, Stary G (* equal contribution)

Disruption of skin homeostasis reveals divergent reconstitution dynamics of human skin tissue-resident memory T cell subsets
(In revision at Nature Immunology)

Most of the research presented in this thesis was conducted at the Department of Medicine I, Medical University of Vienna, Austria, under the supervision of Prof. Sylvia Knapp, MD, PhD, and co-supervised by Mag^a. Riem Gawish, MSc, PhD. Parts of the work were carried out at the Research Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences, Vienna, Austria. All studies involving human samples were performed at the Department of Dermatology, Medical University of Vienna by the laboratory of Prof. Georg Stary, MD, and in compliance with applicable ethical regulations.

The focus of this thesis, as well as my own contributions to the underlying studies, lies in bioinformatic analyses and the application of machine learning frameworks to model immune cell systems. The respective manuscripts provide a transparent account of each co-author's specific role, together with their institutional affiliation. Wet-lab experiments were performed by contributing co-authors with the corresponding experimental expertise. Contributions from core facilities at the Medical University of Vienna and at CeMM are explicitly recognized within the individual publications.

I confirm that all sources have been appropriately acknowledged and that this thesis has not been submitted, in whole or in part, for any other academic degree.

No AI-generated text passages are included in this thesis, apart from the German translation of the English abstract on page vi-vii. This translation was generated using ChatGPT 5.5 and subsequently proofread, revised, and adapted manually. Microsoft Word 365 tools were used for spelling and grammar checks, and ChatGPT 5.5 was used to support paraphrasing and language refinement.

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Abstract (English)

The immune system unfolds in layered waves during embryonic development and remains dynamically adaptable in adult life by reshaping hematopoiesis and immune cell states in response to challenge. Advances in systems immunology and machine learning have helped to establish a view of immunity as a continuum of state transitions, in which local tissue cues and systemic signals jointly remodel hematopoietic and non-hematopoietic compartments. In this framework, disease states are represented within a high-dimensional manifold, offering a way to infer potential interventions that restore homeostasis. This thesis applies these concepts based on two manuscripts that investigate the spleen as a systemic immune niche and the skin as a local barrier organ.

In the mouse spleen, we investigated how inflammatory signals are integrated within the erythroid niche during stress erythropoiesis. By combining time-resolved single-cell RNA sequencing (scRNA-seq), trajectory modeling, and gene regulatory network inference, we identified myeloid type I interferon (IFN-I) sensing as a niche-specific amplifier of splenic stress erythropoiesis. Loss of myeloid IFNAR impaired the transition of recruited monocytes toward red pulp macrophage (RPM) states, marked by reduced SPI-C activity and iron-handling programs. In parallel, erythroid progenitors failed to sustain IFN- and AP-1-associated stress programs, leading to premature activation of erythroblast maturation programs. Together, these findings identify IFN-I as a regulator of RPM development during inflammation and as a niche signal coordinating stress progenitor expansion and terminal differentiation.

In human skin, we investigated immune reconstitution after hematopoietic stem cell transplantation (HSCT) in a longitudinal cohort with and without acute graft-versus-host disease (aGVHD). By integrating scRNA-seq with T cell receptor (TCR) sequencing and single nucleotide polymorphism (SNP) host–donor demultiplexing we resolved T cell states across tissue, time, genotype, and clonotype on single cell level. Donor replacement was rapid in blood but delayed, where donor-derived effector/memory T cells seeded the tissue-resident memory T cell (T_{DRM}) compartment. However, CD8⁺ type 17-polarized T_{DRM} (T_{17DRM}) failed to efficiently reconstitute after HSCT and during aGVHD, while post-transplant skin favored type 1-polarized T_{DRM} (T_{1DRM}) states. Together, these findings identify CD8⁺ T_{17DRM} as a distinct resident T cell population in human skin whose selective reduction after HSCT and during aGVHD may disrupt T_{17DRM}/T_{1DRM} balance and contribute to a pro-inflammatory niche that predisposes to or amplifies cutaneous aGVHD.

Together, these studies provide insight into myeloid and T cell adaptation to inflammation and support a view of inflammatory disease as a failure to coordinate and resolve tissue-specific immune cell state transitions.

Abstract (German)

Das Immunsystem etabliert sich während der Embryonalentwicklung in aufeinanderfolgenden Schüben und bleibt auch im Erwachsenenalter dynamisch anpassungsfähig, indem Hämatopoese und Immunzellzustände als Reaktion auf Herausforderungen umgestaltet werden. Fortschritte in der Systemimmunologie und im maschinellen Lernen haben dazu beigetragen, Immunität als ein Kontinuum zellulärer Zustandsübergänge zu verstehen, in dem lokale Gewebesignale und systemische Signale gemeinsam hämatopoetische und nicht-hämatopoetische Kompartimente regulieren. In diesem Rahmen werden Krankheitszustände innerhalb einer hochdimensionalen Mannigfaltigkeit dargestellt, wodurch potenzielle Interventionen abgeleitet werden können, die zur Wiederherstellung der Homöostase beitragen. Diese Dissertation vergleicht diese Konzepte anhand zweier Studien, wobei einerseits die Milz als systemische Immunische und andererseits die Haut als lokales Barriereorgan untersucht werden.

In der Mausmilz untersuchten wir, wie inflammatorische Signale während der Stress-Erythropoese innerhalb der erythroiden Nische integriert werden. Durch die Kombination von zeitaufgelöster Einzelzell-RNA-Sequenzierung (scRNA-seq), Trajektorienmodellierung und Inferenz genregulatorischer Netzwerke identifizierten wir die myeloide Typ-I-Interferon-(IFN-I)-Erkennung als nischenspezifischen Verstärker der Stress-Erythropoese in der Milz. Die Abwesenheit von myeloidem IFNAR beeinträchtigte den Übergang rekrutierter Monozyten zu roten Pulpa-Makrophagen (RPM), gekennzeichnet durch reduzierte SPI-C-Aktivität und abgeschwächte Eisenstoffwechselprogramme. Parallel dazu konnten IFN- und AP-1-assoziierte Stressprogramme in erythroiden Vorläuferzellen nicht aufrechterhalten werden, was zu einer vorzeitigen Aktivierung von Erythroblasten-Reifungsprogrammen führte. Zusammen identifizieren diese Befunde IFN-I als Regulator der RPM-Entwicklung während der Entzündung und als Nischensignal, das die Expansion von erythroiden Stress-Vorläuferzellen mit der terminalen Differenzierung koordiniert.

In der humanen Haut untersuchten wir die Immunrekonstitution nach hämatopoetischer Stammzelltransplantation (HSCT) in einer longitudinalen Kohorte mit und ohne akuter Graft-versus-Host-Erkrankung (aGVHD). Durch die Integration von scRNA-seq mit T-Zell-Rezeptor-(TCR)-Sequenzierung und SNP-basierter Wirt-Spender-Demultiplexierung konnten wir T-Zell-Phänotypen über Gewebe, Zeit, Genotyp und Klonotyp hinweg auf Einzelzellebene auflösen. Die Rekonstituierung durch Spenderzellen erfolgte im Blut rasch, war jedoch in der Haut verzögert, wo neue Effektor-/Gedächtnis-T-Zellen des Spenders das Kompartiment geweberesidenten Gedächtnis-T-Zellen (TD_RM) besiedelten. CD8⁺ Typ-17-polarisierte TD_RM (T₁₇TD_RM) rekonstituierten sich jedoch nach HSCT und während aGVHD nur unzureichend, während die Haut nach Transplantation Typ-1-polarisierte TD_RM-Zustände (T₁TD_RM)

begünstigte. Zusammen identifizieren diese Ergebnisse CD8⁺ T₁₇D_RM als distinkte residente T-Zell-Population der humanen Haut. Die selektive Reduktion von CD8⁺ T₁₇D_RM nach HSCT und während aGVHD kann das Gleichgewicht zwischen T₁₇D_RM und T₁D_RM stören und ein proinflammatorisches Mileu in der Nische bewirken, was die kutane aGVHD begünstigt oder verstärkt.

Zusammen liefern diese Studien Einblicke in die Anpassung myeloider Zellen und T-Zellen an Entzündung und unterstützen ein Verständnis inflammatorischer Erkrankungen als Störung der koordinierten Auslösung und Auflösung gewebespezifischer Immunzell-Zustandsübergänge.

Publications arising from this thesis

The research conducted during my doctoral training has resulted in two primary publications, which are included in this thesis in accordance with the declaration of authorship:

Deckert F, Hladik A, Lakovits K, Farhat A, Pimenov L, Meng F, Fokina A, Quattrone F, Gorki AD, Gawish R, Knapp S

Myeloid IFN-I sensing boost splenic stress erythropoiesis
(Submitted to Blood)

Gail LM*, **Deckert F***, Thébault L, Dingelmaier-Hovorka R, Strobl J, Eskandari D, Kleissl L, Reininger B, Mayerhofer C, Gabriel A, Freystätter C, Shaw L, Rüsing L, Cserna J, Weninger W, Farlik M, Rabitsch W, Strydom G (* equal contribution)

Disruption of skin homeostasis reveals divergent reconstitution dynamics of human skin tissue-resident memory T cell subsets
(In revision at Nature Immunology)

In addition, I contributed to or lead bioinformatics on several collaborative research projects during my PhD, leading to co-authorships on further peer-reviewed publications. These works are not formally incorporated into this thesis but include the application of computational concepts and methods to model immune systems.

Gawish R, Varada R, **Deckert F**, Hladik A, Steinbichl L, Cimatti L, Milanovic K, Jain M, Torgasheva N, Tanzer A, De Paepe K, Van de Wiele T, Hausmann B, Lang M, Pechhacker M, Ibrahim N, De Vries I, Brostjan C, Sixt M, Gasche C, Boon Ö, Berry D, Jantsch MF, Pereira FC, and Vesely C

Filamin A editing in myeloid cells reduces intestinal inflammation and protects from colitis
Journal of Experimental Medicine 2025 Sep 1;222 (9):e20240109

Farhat A, Radhouani M, **Deckert F**, Zahalka S, Pimenov L, Fokina A, Hakobyan A, Oberndorfer F, Brösamlen J, Hladik A, Lakovits K, Meng F, Quattrone F, Boon L, Vesely C, Starkl P, Boucheron N, Menche J, Van der Veecken J, Ellmeier W, Gorki AD, Campbell C, Gawish R*, Knapp S* (*shared last authorship)

An aging bone marrow exacerbates lung fibrosis by fueling profibrotic macrophage persistence
Science Immunology 2025 Mar 28; 10(105):eadk5041

Strydom V, Pandey RV, List J, Kleissl L, **Deckert F**, Kabiljo J, Laengle J, Gerakopoulos V, Oehler R, Watzke L, Farlik M, Lukowski SW, Vogt AB, Strydom G, Stockinger H, Bergmann M, Pilat N
Dysfunctional tumor-infiltrating $\gamma\delta 1+$ T lymphocytes in microsatellite-stable colorectal cancer
Nature Communications 2024 Aug 13;15(1):6949

Bayer N, Hausmann B, Pandey RV, **Deckert F**, Gail LM, Strobl J, Pjevac P, Krall C, Unterluggauer L, Redl A, Bachmayr V, Kleissl L, Nehr M, Kirkegaard R, Makristathis A, Watzenboeck ML, Nica R, Staud C, Hammerl L, Wohlfarth P, Ecker RC, Knapp S, Rabitsch W, Berry D, Stary G

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

Leukemia 2022 Nov;36(11):2705-2714

Zahalka S, Starkl P, Watzenboeck ML, Farhat A, Radhouani M, **Deckert F**, Hladik A, Lakovits K, Oberndorfer F, Lassnig C, Strobel B, Klavins K, Matsushita M, Sanin DE, Grzes KM, Pearce EJ, Gorki AD*, Knapp S* (*shared last authorship)

Trained immunity of alveolar macrophages requires metabolic rewiring and type 1 interferon signaling

Mucosal Immunology 2022 May;15(5):896-907

In addition, I contributed computational analysis to several manuscripts that have either been submitted or are currently under review. Among others, these forthcoming publications are not considered part of the present thesis:

Fokina A, Pimenov L, Hladik A, Lakovits K, Farhat A, **Deckert F**, Radhouani M, Broesamlen J, Meng F, Gawish R* & Knapp S* (*shared last authorship)

Early and late pregnancy differentially impact maternal alveolar macrophages' function and immunity to SARS-CoV-2 infection

(Under revision at Mucosal Immunology)

Winkler F, Fischer C, Gail LM, **Deckert F**, Kleissl L, Krausgruber T, Brazdilova K, González AA, Stary G, Strobl J

Virus-specific resident memory T cell networks sustain immunity in human oral mucosa

(Under revision at Mucosal Immunology)

Pimenov L, **Deckert F**, Fokina A, Farhat A, Radhouani M, Broesamlen J, Fanzhe M, Hladik A, Lakovits K, Salzer I, Schicker K, Repic A, Starkl P, Gawish R*, Knapp S* (*shared last authorship)

Passenger mutations in epithelial cells can autonomously determine outcomes of respiratory viral infections

(Submitted)

Abbreviations

Abbreviation	Full form
AGM	Aorto-gonad-mesonephros region
AIM2	Absent in melanoma 2
ALR	AIM2-like receptor
AP-1	Activator protein 1
APC	Antigen-presenting cell
BCR	B cell receptor
BFU-E	Burst-forming unit erythroid
cDC1	Type 1 conventional dendritic cell
cDC2	Type 2 conventional dendritic cell
CDP	Common dendritic progenitor
cGAS	Cyclic GMP-AMP synthase
cGVHD	Chronic graft-versus-host disease
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
CMF	Common myeloid progenitor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DETC	Dendritic epidermal T cell
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
E	Embryonic day
EC	Endothelial cell
EHT	Endothelial-to-hematopoietic transition
EMP	Erythro-myeloid progenitor
GMP	Granulocyte-monocyte progenitor
GVHD	Graft-versus-host disease
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cell
IFN- γ	Interferon- γ
IFN-I	Type I interferon
IFNAR	Type I interferon receptor
Ig	Immunoglobulin

Abbreviation	Full form
IL	Interleukin
ILC	Innate lymphoid cell
iNKT	Invariant natural killer T cell
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
JAK1	Janus kinase 1
LT-HSC	Long-term hematopoietic stem cell
LT β R	Lymphotoxin- β receptor
LTi	Lymphoid tissue inducer cell
LTo	Lymphoid tissue organizer cell
MAIT	Mucosal-associated invariant T cell
MDP	Monocyte- and dendritic-cell-biased progenitor
MEP	Megakaryocyte-erythroid progenitor
MHC	Major histocompatibility complex
MICA	MHC class I chain-related gene A
MPP	Multipotent progenitor
MZ	Marginal zone
MZM	Marginal zone macrophage
NF- κ B	Nuclear factor κ B
NK	Natural killer cell
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PRR	Pattern-recognition receptor
RAG	Recombination-activating gene
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RPM	Red pulp macrophage
S1P	Sphingosine-1-phosphate
S1PR1	Sphingosine-1-phosphate receptor 1
SCF	Stem cell factor
scRNA-seq	Single-cell RNA sequencing
scVI	Single-cell variational inference

Abbreviation	Full form
SLO	Secondary lymphoid organ
ssRNA	Single-stranded RNA
ST-HSC	Short-term hematopoietic stem cell
STAT	Signal transducer and activator of transcription
T17DRM	Type 17-polarized tissue-resident memory T cell
T1DRM	Type 1-polarized tissue-resident memory T cell
TBM	Tingible body macrophage
TCM	Central memory T cell
TCR	T cell receptor
TDexRM	TDRM-derived ex-resident memory T cell
TDRM	Tissue-resident memory T cell
TEM	Effector memory T cell
TFH	T follicular helper cell
TGF- β	Transforming growth factor- β
TH	T helper cell
TIR	Toll/IL-1 receptor domain
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNF- α	Tumor necrosis factor- α
Treg	Regulatory T cell
TRIF	TIR-domain-containing adaptor-inducing interferon- β
TRM	Tissue-resident macrophage
TYK2	Tyrosine kinase 2
YS	Yolk sac

Acknowledgments

In the end, we all roll down the Waddington landscape of life, shaped by our environment, chance, and the occasional perturbation. This thesis would not have been possible without the unconditional support, patience, and love of my family. I am immensely grateful to my mother, Petra Steiner-Deckert, my father, Feri Deckert, and my stepfather, Michael Steiner. I also want to thank my grandparents, Fritz and Renate Wölfel as well as Franz and Maria Deckert, for being outstanding role models and for shaping the landscape on which I started my journey. A very special thanks goes to my little sister, Karina Hamacher, who may be younger, but is undoubtedly the true giant of the family.

While the PhD has been an intellectually fulfilling journey, its importance is easily dwarfed by the joy, chaos, and wonder brought into my life by my daughters, Louise Isidora Deckert and Paula Maxima Deckert. I am especially grateful to my partner, Susanne Hofmarcher, whose love, patience, and support carried me through the most demanding parts of this journey and made the good moments even brighter. While we both followed our career paths, I am immensely grateful to Sabine and Herta Hofmarcher for always being there for our children and for supporting us along the way.

As mentioned above, our journey partly depends on chance, and I am incredibly thankful to Sylvia Knapp for taking me on as a PhD student and giving me exactly that: a chance. Working in her laboratory was, without question, a scientifically outstanding experience. However, I am even more grateful that she believed in me and consistently reminded me that life is not only governed by randomness, but that I am responsible for shaping my own path. This has been one of the most important lessons of my PhD, and it helped me grow far beyond the scientific work itself.

On this note, I am deeply grateful to my co-supervisor, Riem Gawish, who has been my principal scientific role model. Her knowledge, creativity, and scientific intuition helped me grow beyond simply crunching numbers and taught me to fully appreciate the complexity and beauty of immunology.

I am immensely grateful to all my colleagues in Sylvia Knapp's laboratory, who supported me with their work, advice, and friendship over the years. I would like to thank Martin Watzenböck, Lisabeth Pimenov, Asma Farhat, Sophie Zahalka, Karin Lakovits, and Anastasiya Hladik explicitly for making the lab such a supportive and inspiring place.

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the second manuscript presented in this thesis. Thank you so much, Laura, for giving me this opportunity and for being a dear friend.

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Finally, I would like to thank all the people at the Medical University of Vienna (MUW) and the Research Center for Molecular Medicine (CeMM) who supported me with organizational tasks and running the research facilities. Their work was indispensable for the research presented in this thesis.

1 Introduction

This thesis investigates the immune system as a developmentally continuous network of cells whose functional states are shaped by the need to maintain tissue integrity while adapting to inflammatory challenges.

The manuscripts included in this thesis examine two complementary models of inflammation-driven immune remodeling. In the mouse spleen, we investigated how systemic inflammatory cues regulate the differentiation of monocyte-derived red pulp macrophages (RPMs) and the role of myeloid IFN-I sensing in shaping the erythroid niche during stress erythropoiesis. In the human skin, we studied T cell tissue residency following allogeneic hematopoietic stem cell transplantation (HSCT) and during acute graft-versus-host disease (aGVHD), addressing how inflammatory environments influence the establishment, maintenance, and functional polarization of tissue-resident memory T cells. Together, these projects explore how inflammation restructures both lymphoid and myeloid compartments, linking tissue-resident immune adaptation to hematopoietic remodeling under stress conditions. Chapter 1.1 introduces the immune system as a developmental continuum, outlining key principles of hematopoiesis and how tissue context shapes immune cell identity. Chapter 1.2 introduces inflammation as a major driver of hematopoietic and immune remodeling. Chapters 1.3 and 1.4 then examine two complementary immune niches, the spleen as a systemic immune organ and the skin as a local barrier tissue. Finally, Chapter 1.5 introduces computational frameworks for representing and interpreting immune cell states within a dynamic and adaptive system.

By integrating immunological principles with high-dimensional single-cell profiling and computational modeling approaches, this thesis aims to define how tissue microenvironment and inflammatory perturbation collectively shape macrophage and T cell function.

1.1 The immune system as a developmental and functional continuum

In vertebrates, hematopoiesis unfolds through at least three partially overlapping developmental waves that arise during prenatal development at distinct anatomical sites and are later maintained by hematopoietic stem cells (HSCs) residing in specialized stromal niches (Canu & Ruhrberg, 2021; Goh et al., 2023; Orkin & Zon, 2008). Each wave contributes to the establishment of **tissue-resident** immune populations that self-maintain locally and play essential roles in tissue homeostasis and immune defense (J. Li et al., 2025). Therefore, each wave contributes to a new layer of the emerging immune system, and each layer parallels the evolutionary emergence of the respective immune cell types. This observation was first conceptualized by Leonore A. and Leonard A. Herzenberg in 1986 and termed the **layered immune system** (Herzenberg & Herzenberg, 1989). A hematopoietic **lineage** refers to the developmental ancestry of an immune cell. It captures the sequence of cell states that connect a progenitor to its mature descendants. Lineage relationships define broad organizational

principles of the immune system yet do not fully determine functional behavior. As progenitors progress along a lineage, they pass through phases of commitment and differentiation, two related but mechanistically distinct processes. **Commitment** denotes the initial restriction of developmental potential. At this stage, a stem or progenitor cell becomes biased toward a defined lineage through activation of lineage-associated transcriptional programs and suppression of alternative developmental potential. Early commitment may retain partial plasticity, particularly in intermediate progenitor states where competing regulatory circuits have not yet been fully extinguished. In contrast, differentiation represents the subsequent and largely irreversible execution of a lineage program. During **differentiation**, cells consolidate identity through stable transcriptional and epigenetic reconfiguration and acquire the structural and functional characteristics of a specialized immune cell type. Following differentiation, many immune cells undergo **maturation**, during which they acquire full functional competence. This process typically occurs within specialized anatomical sites, such as primary and secondary lymphoid organs or peripheral tissues, where defined microenvironments provide instructive signals. In contrast to commitment and differentiation, which establish lineage identity, **polarization** describes the context-dependent functional adaption of mature immune cells. Polarization is driven by environmental cues such as cytokines, antigen exposure, or tissue-derived signals and modulates effector programs. These polarized states are dynamic and often reversible, allowing immune cells to adjust their functional profile to physiological or inflammatory conditions.

1.1.1 Hematopoiesis and the organization of the immune system

In mice, the **first hematopoietic wave** arises around embryonic day (E) 7.0 in the extraembryonic yolk sac (YS) and is intimately linked to vascular development (Figure 1) (Canu & Ruhrberg, 2021). Mesoderm-derived endothelial and hematopoietic progenitors progressively organize in blood islands (E8.5) that give rise to primitive erythroblasts, megakaryocytes, and macrophages which subsequently start migrating into the embryo (Ferkowicz & Yoder, 2005). Primitive erythroblasts are large, nucleated cells that express embryonic hemoglobins required for early oxygen transport and undergo enucleation later within the embryo (Fraser et al., 2007; Ji et al., 2003; Kingsley et al., 2004). In parallel, primitive macrophages develop in a c-Myb-independent manner and without a monocyte intermediate. Primitive macrophages that invade the embryo constitute the lifelong predominant source of microglia, the tissue-resident macrophages of the central nervous system (Ginhoux et al., 2010; McGrath, Frame, & Palis, 2015).

The **second hematopoietic wave** of fetal YS hematopoiesis originates from the vascular hemogenic endothelium. Through endothelial-to-hematopoietic transition (EHT) endothelial cells give rise to erythro-myeloid progenitors (EMPs) (Kasaai et al., 2017). EMPs migrate to

the fetal liver to expand and differentiate into definitive erythroid, megakaryocyte, macrophages (Guan et al., 2025), eosinophils (Voehringer et al., 2007), neutrophils (McGrath, Frame, Fegan, et al., 2015), mast cells (Gentek et al., 2018; Z. Li et al., 2018). Unlike primitive macrophage progenitors, EMPs are Myb-dependent and generate fetal monocytes that seed multiple tissues before birth (Gomez Perdiguero et al., 2015). EMP-derived fetal monocytes give rise to most tissue-resident macrophages (TRM) outside the brain, including Kupffer cells in the liver, Langerhans cells in the epidermis, alveolar macrophages in the lung, and red pulp macrophages in the spleen.

Mouse developmental stages

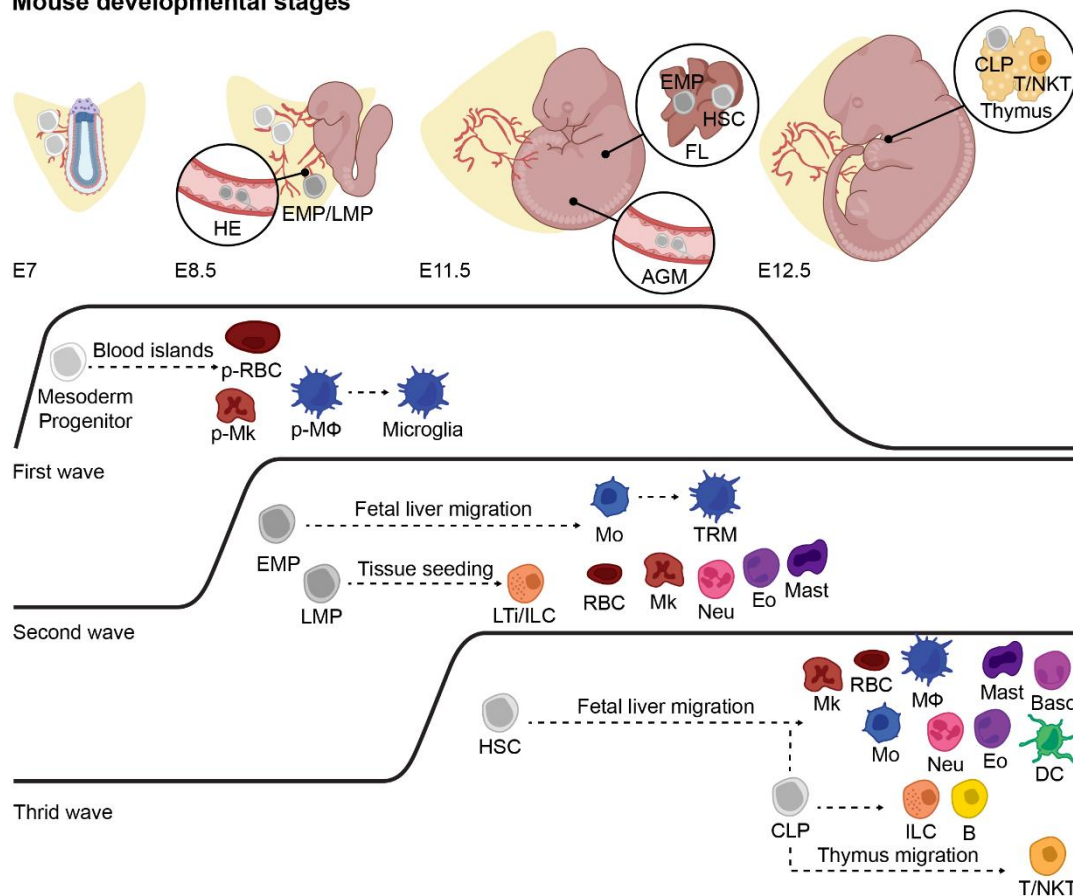


Figure 1 The three waves of the developing immune system.

Schematic illustration of the developing immune system in the mouse, organized into three partially overlapping hematopoietic waves and highlighting the developmental origin of progenitors and the major anatomical sites of hematopoiesis. Abbreviations: AGM, aorta–gonad–mesonephros; CLP, common lymphoid progenitor; EMP, erythro-myeloid progenitor; FL, fetal liver; HE, hemogenic endothelium; HSC, hematopoietic stem cell; ILC, innate lymphoid cell; LMP, lympho-myeloid progenitor; LTI, lymphoid tissue inducer cell; p-MΦ, primitive macrophage; p-Mk, primitive megakaryocyte; p-RBC, primitive red blood cell; TR-MΦ, tissue-resident macrophage.

Thus, most adult tissue macrophage compartments are established prenatally from YS-derived progenitors rather than from definitive HSCs. Further, definitive macrophages in the fetal liver assemble erythroblastic islands (May & Forrester, 2020) that maintain iron recycling and phagocytic functions to sustain definitive erythropoiesis (X.-S. Liu et al., 2007; Porayette &

Paulson, 2008; Spike et al., 2007; Lina Wang et al., 2017). In addition, these macrophages promote the terminal maturation and clearance of remaining nuclei from circulating primitive erythroblasts (Fraser et al., 2007; Isern et al., 2008; Kingsley et al., 2004). By E16.5, primitive erythroblasts are almost entirely replaced by liver-derived definitive erythrocytes (Fraser et al., 2007). The YS also generates early lymphoid progenitors with innate potential and their contribution can persist into adulthood (Kobayashi et al., 2023; Ni et al., 2024; Palacios & Imhof, 1993; Patel et al., 2022; Yokomizo & Suda, 2024). Progenitors from the hemogenic endothelium give rise to the majority of lymphoid tissue inducer (LTi) cells in the fetal liver, a ROR γ t⁺ group 3 innate lymphoid cell (ILC) subset (Lane et al., 2009; Simic et al., 2020; Spits et al., 2013; van de Pavert, 2023). These embryonic LTi cells accumulate at sites of future lymph nodes (E12.5-E13.5) and Preyer's patches (E15.5-E16.5) where they cluster with mesenchymal lymphoid tissue organizer (LTo) cells. Through membrane-bound lymphotoxin (LT α 1 β 2) signaling via LT β R on LTo cells, LTi cells induce expression of CXCL13, CCL19, and CCL21, thereby promoting stromal activation, hematopoietic cell recruitment, and the structured assembly of secondary lymphoid organs (van de Pavert & Mebius, 2010). Recent work further indicates that YS might give rise to B-1a B cells and selected embryonic $\gamma\delta$ T cell subsets (Tan et al., 2021; Tieppo et al., 2020; Vermijlen & Prinz, 2014) independent of definitive HSC intermediate (Hadland & Yoshimoto, 2018; Ni et al., 2024).

The **third hematopoietic wave** generates the first long-term repopulating HSCs (E8.5 until E11.5) through endothelial-to-hematopoietic transition (EHT) of hemogenic endothelium in the major embryonic arteries, most prominently the dorsal aorta within the aorto-gonad mesonephros AGM region (Boisset et al., 2010). Nascent HSCs arise in intra-aortic hematopoietic clusters and acquire durable self-renewal and multilineage differentiation capacity, a property that distinguishes them from early yolk-sac-derived hematopoietic progenitors (Cain et al., 2025). Following expansion in the fetal liver, definitive hematopoiesis generates the full spectrum of blood cells of the myeloerythroid (erythrocytes, megakaryocytes, monocytes, dendritic cells, neutrophils, eosinophils, basophils, mast cells) and lymphoid (innate lymphoid cells, B lymphocytes, and T lymphocytes) lineage. Although innate lymphoid cells share a common lymphoid progenitor (CLP) with B and T lymphocytes, they belong to the innate immune system and lack somatically rearranged antigen receptors. CLP-derived progenitors give rise to LTi as well as to cytotoxic natural killer (NK) cells and cytokine-producing helper-like subsets (ILC1, ILC2, and ILC3). Both NK cells and ILCs seed barrier tissues and mucosal sites during embryogenesis, preceding the full establishment of adaptive immunity (Gasteiger et al., 2015; J. Li et al., 2025; Spits et al., 2013). Common lymphocyte progenitors seed and mature in the thymus around E11-12, which develops from the third pharyngeal pouch endoderm. During thymic development, thymocytes progress through CD4-CD8- double-negative stages, where RAG1/2-mediated V(D)J recombination assembles the T

cell receptor (TCR). Productive TCR β rearrangement triggers β -selection and proliferation, allowing differentiation into CD4⁺ CD8⁺ double-positive thymocytes where TCR α recombination occurs. Subsequent positive and negative selection generate mature CD4⁺ and CD8⁺ $\alpha\beta$ T cells that exit the thymus as naïve lymphocytes and circulate through secondary lymphoid organs (Sun et al., 2023). Conventional tissue-resident memory T cells (T_{RM}) do not arise during thymic development but are generated later from activated effector T cells following antigen encounter in peripheral tissues, where they adopt long-term residency programs. In contrast, several innate-like T cell lineages including $\gamma\delta$ T cells, invariant natural killer T (iNKT) cells, and mucosal-associated invariant T (MAIT) cells can acquire tissue-biased localization soon after thymic development (Godfrey et al., 2015). These populations typically express semi-invariant TCRs with restricted repertoire diversity, respond rapidly to stimulation, and often recognize non-peptide antigens presented by non-classical antigen-presenting molecules such as CD1d or MR1, thereby providing fast effector responses that functionally bridge innate and adaptive immunity. From around E12.5, the first B-lymphocyte progenitors become detectable in the fetal liver, where they progress through pro-B and pre-B stages characterized by sequential immunoglobulin heavy- and light chain V(D)J recombination, ultimately generating immature IgM⁺ B cells. These cells subsequently migrate to peripheral lymphoid organs, particularly the spleen, where they mature through transitional stages into functionally distinct B cell subsets. Early in development, B cell output is biased toward the innate-like B-1 lineage (B-1a and B-1b), which displays restricted B cell receptor diversity and constitutively produces natural IgM antibodies that provide rapid first-line humoral defense. Mature B-1 cells preferentially populate serous cavities, such as the peritoneal and pleural spaces, where they persist as long-lived self-renewing populations. Conventional B-2 cells, which mediate adaptive humoral immunity, arise later and differentiate in the spleen into follicular B cells and marginal-zone B cells that participate in germinal-center responses and antibody diversification. This layered developmental program is particularly pronounced in mice, where fetal progenitors efficiently generate B-1 cells, whereas in humans the B-1 lineage is less clearly defined and fetal B-lymphopoiesis contributes predominantly to conventional B-2 cells that populate secondary lymphoid organs. Around E16.5 definitive HSC that have expanded in the fetal liver begin migrating to the developing bone marrow, which progressively becomes the principal site of lifelong hematopoiesis (Ganuza et al., 2022; Gao et al., 2018). This colonization marks a developmental transition from transient embryonic hematopoietic sites to a permanent, highly structured adult niche. While fetal liver hematopoiesis is characterized by rapid expansion and a high proliferative output, the bone marrow environment enforces a distinct regulatory state in which HSC quiescence, long-term self-renewal, and balanced multilineage differentiation are tightly controlled.

1.1.2 Transcriptional control of lineage priming in adult hematopoiesis

Classical models of hematopoiesis were largely informed by colony-forming assays, which defined progenitor populations based on their *in vitro* differentiation potential and thereby supported a hierarchical, tree-like organization of lineage relationships (Yusoff et al., 2025). However, these approaches capture functional potential under defined conditions rather than *in vivo* developmental trajectories. Recent single-cell and lineage-tracing studies have refined this framework by revealing continuous transcriptional landscapes and early lineage priming, suggesting that hematopoiesis is more accurately described as a **dynamic continuum** rather than as a series of discrete branching events, as depicted in Figure 2 (Ferchen et al., 2025; Liggett & Sankaran, 2020; Safina & van Galen, 2024). In the following, lineage relationships are described using transcription factor-associated progenitor states as a conceptual framework.

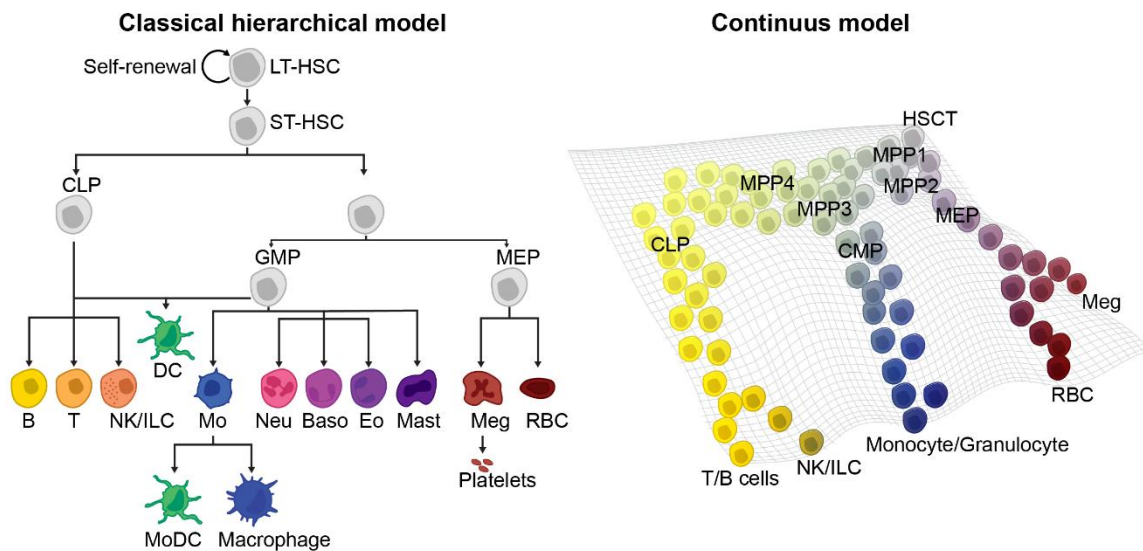


Figure 2 Hierarchical and continuous models of adult hematopoiesis

Schematic representation of adult hematopoiesis as a classical hierarchical model and as a continuous differentiation landscape. The hierarchical model summarizes major progenitor compartments and lineage relationships, whereas the continuous model emphasizes gradual lineage priming and transcriptional state transitions within the hematopoietic compartment. Abbreviations: B, B cell; Baso, basophil; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; Eo, eosinophil; GMP, granulocyte–monocyte progenitor; HSC, hematopoietic stem cell; LT-HSC, long-term HSC; Mast, mast cell; Meg, megakaryocyte; MEP, megakaryocyte–erythroid progenitor; Mo, monocyte; MoDC, monocyte-derived dendritic cell; MPP, multipotent progenitor; Neu, neutrophil; NK/ILC, natural killer cell/innate lymphoid cell; RBC, red blood cell; ST-HSC, short-term HSC; T, T cell.

In the adult bone marrow, HSCs reside within perivascular niches maintained by endothelial cells (ECs) and mesenchymal stromal cells (MSCs). Stem cell factor (SCF) produced by these cells activates c-KIT signaling to sustain HSC survival and functional competence, whereas NOTCH signaling and CXCL12-CXCR4 interactions mediate quiescence and niche retention, respectively (Comazzetto et al., 2021). HSC are enriched within the lineage-negative Sca-1+

c-Kit⁺ (LSK) compartment, and two functionally distinct subsets can be distinguished (Spangrude et al., 1988). **Long-term HSCs (LT-HSCs)** LSK CD150⁺ CD48⁻ CD34⁻ Flt3⁻ constitute a rare population with durable self-renewal, whereas **short-term HSCs (ST-HSCs)** LSK CD150⁺ CD48⁻ CD34⁺ Flt3⁻ represent a downstream transitional state with limited self-renewal and enhanced proliferative output (Kiel et al., 2005). LT-HSC maintenance is reinforced by hypoxia-adapted metabolic programs that restrict mitochondrial activity and limit reactive oxygen species accumulation. Additional hematopoietic (megakaryocytes, macrophages) and non-hematopoietic (osteolineage cells, Schwann cells, and adipocytes) population further modulate the niche environment, contributing to spatial heterogeneity in cellular composition and cytokine availability (TGF- β and CXCL4). While this structural diversity clearly influences HSC activation and mobilization dynamics, the extent to which distinct niche configurations directly bias long-term lineage fate remains an area of active investigation (Hofmann & Kokkaliaris, 2024; Meng & Nerlov, 2025). While LT-HSCs are in principle capable of reconstituting all blood cell lineages (Kiel et al., 2005; McCulloch & Till, 1960; Osawa et al., 1996; Spangrude et al., 1988) recent advances in clonal tracing and single-cell transcriptomics have revealed LT-HSC lineage bias in mice and humans (Jacobsen & Nerlov, 2019; Yoshizato et al., 2025). In the unchallenged bone marrow, at least three LT-HSC subsets with exclusive megakaryocyte, megakaryocyte-erythroid-myeloid, or multilineage potential have been identified (Carrelha et al., 2018). The myeloerythroid-restricted reconstitution bias of LT-HSC and the absence of lymphoid-restricted LT-HSC subsets are compatible with the peak production of lymphoid progenitors observed during fetal development and the neonatal period (Carrelha et al., 2018; Dykstra et al., 2007; Müller-Sieburg et al., 2002). **Multipotent progenitors (MPPs)** arise downstream of short-term HSCs and are characterized by high proliferative capacity while lacking long-term self-renewal. MPPs are subdivided into MPP1-MPP4 states with distinct lineage biases, ranging from immature stem-like compartments (MPP1) to megakaryocyte-erythroid (MPP2), myeloid (MPP3), or lymphoid (MPP4) primed progenitors (Pietras et al., 2015). Importantly, these progenitor states do not represent fixed developmental intermediates but instead reflect a continuum of lineage bias shaped by both intrinsic transcriptional programs and extrinsic signals. Downstream of MPPs, **common myeloid progenitor (CMP)** and **common lymphoid progenitor (CLP)** are classically defined, although lineage boundaries at this stage remain still permeable rather than absolute. Megakaryocyte-erythroid differentiation is commonly associated with a **megakaryocyte-erythroid progenitor (MEP)** state and is reinforced by antagonism of PU.1 through a shift from GATA2- to GATA1-dominant regulation (Galloway et al., 2005). Myeloid differentiation is often represented through a **granulocyte-monocyte progenitor (GMP)** state and is strongly influenced by PU.1 dosage and combinatorial transcription factor activity (Mak et al., 2011). PU.1 expression together with C/EBP α favors the development of granulocytes

(Pundhir et al., 2018). Notably, basophil- and mast cell-biased progenitors display reduced reliance on C/EBP α compared to neutrophil or eosinophil committed cells and retain residual megakaryocyte-erythroid differentiation potential, consistent with an earlier divergence from other myeloid fates (Wanet et al., 2021). High PU.1 activity together with IRF8 is associated with **monocyte- and dendritic-cell-biased progenitor (MDP)** states downstream of GMP (Kurotaki et al., 2013). Monocyte differentiation is further reinforced by the IRF8–KLF4 axis and by MAFB, while dendritic-cell identity remains associated with sustained IRF8 activity and FLT3 expression (Kurotaki et al., 2013). **Common dendritic progenitors (CDP)** give rise to classical dendritic cells 1 (cDC1), classical dendritic cells 2 (cDC2), and plasmacytoid dendritic cells (pDCs). Notably, pDCs can arise from both, CDP and CLP (Xie et al., 2025). Within CLP-like compartments, lymphoid progenitors must first exit the bone marrow and home to the thymus before **T cell lineage** specification can proceed. Bone marrow retention is maintained in part by CXCL12–CXCR4 signaling, whereas thymus-seeding progenitors acquire migratory competence through CCR7- and CCR9-dependent chemotaxis together with PSGL1-mediated interactions with thymic P-selectin (Rossi et al., 2005; Zlotoff et al., 2010). After thymic entry, sustained NOTCH1 signaling induces TCF1 and GATA3, followed by activation of BCL11B, which consolidates T cell lineage commitment and represses innate lymphoid potential (Van de Walle et al., 2016). By contrast, **B cell lineage** commitment is initiated in the bone marrow through sequential activation of EBF1 and PAX5, which progressively stabilize B-lineage identity and extinguish alternative developmental programs (Nutt & Kee, 2007). **Innate lymphoid cell** specification, in turn, proceeds largely extrathymically through NFIL3 and TOX and is consolidated by ID2, which suppresses E-protein-dependent adaptive lymphocyte programs and stabilizes innate lymphoid identity. (Zook et al., 2018).

1.1.3 Tissue context as a determinant of immune cell identity

While hematopoiesis defines what immune cells can become, tissue context determines what they are. Cells of the same lineage can adopt markedly different phenotypes depending on the tissue environment in which they reside. Tissue context integrates spatial organization, stromal and vascular interactions, cytokine milieus, and metabolic constraints to stabilize tissue specific cellular programs. A central manifestation of tissue identity is the establishment of long-lived resident cell populations. While multiple immune cell lineages can adopt tissue residency, this thesis focuses on tissue-resident macrophages and tissue-resident memory T cells.

The ontogeny of tissue macrophages in general was long interpreted through the framework of van Furth's **mononuclear phagocyte system** (van Furth & Cohn, 1968), which proposed that circulating blood monocytes continuously replenished macrophages in peripheral tissues. This view was strongly supported by irradiation chimera and adoptive transfer experiments, in

which donor-derived bone marrow cells efficiently repopulated macrophage niches after depletion. Only later did it become clear that these models had largely measured macrophage replacement under conditions of artificial niche vacancy rather than physiological homeostasis (Ginhoux et al., 2010; Merad et al., 2002; Murphy et al., 2008). Subsequent parabiosis and fate-mapping studies fundamentally revised this concept by demonstrating that macrophage ontogeny is tissue-dependent, revealing that many **tissue-resident macrophages** are established prenatally from embryonic precursors and maintained locally throughout adult life (Hashimoto et al., 2013; Yona et al., 2013). Consequently, it was established that microglia, alveolar macrophages, Langerhans cells, and red pulp macrophages exhibit minimal turnover from circulating monocytes, whereas macrophages in the gut, dermis and heart show a substantially greater contribution from monocytes even under steady-state conditions. While tissue-resident macrophages are important for immune defense, their tissue-specific functions often include unique roles in homeostasis (Zhao et al., 2024). However, through the lifetime of an individual inflammatory signals drive the replacement of embryonically derived tissue-resident macrophages (TRMs) by monocyte-derived TRMs (mo-TRMs). The extent to which mo-TRMs fully recapitulate the transcriptional, epigenetic, and functional identity of embryonic TRMs, as well as the precise niche-derived signals governing this process is an active area of investigation (Aegerter et al., 2020; Farhat et al., 2025).

While tissue-resident macrophages are seeded during embryonic development, **tissue-resident memory T cells** (T_{DRM}) are established postnatally following antigen-driven activation in peripheral tissues. Given the conceptual complexity of T cell subset definitions, this thesis adopts the most recent consensus guidelines for T cell nomenclature from here on forward (J. Li et al., 2025). Naïve T cells are primed by professional antigen-presenting cells (APCs), primarily DCs, within secondary lymphoid organs such as lymph nodes and the spleen, where processed antigen is presented in the context of peptide–MHC together with co-stimulatory signals and inflammatory cytokines. This priming event drives clonal expansion and differentiation into effector populations, the majority of which are short-lived and die during the contraction phase, whereas a subset survives and transitions into long-lived memory T cells. Early models assumed that these memory T cells primarily recirculate through the blood and secondary lymphoid organs, where they persist in a quiescent state awaiting secondary antigen exposure. However, this view has been fundamentally revised by the identification of T_{DRM} in the gut (Masopust et al., 2006) and skin (Gebhardt et al., 2009; X. Jiang et al., 2012). Initially it was believed that T_{DRM} are restricted to patrol their tissue microenvironment and represent terminal states. This view was subsequently challenged by evidence that a subset of T_{DRM} can exit peripheral tissues, re-enter the circulation, and seed distant anatomical sites, a process referred to as **retrograde migration** (Behr & Parga-Vidal, 2020; Beura et al., 2018). These findings led to the concept of **outside-in immunity**, emphasizing the role of peripheral

tissues as active sites of immune initiation, where resident T cells can contribute to rapid systemic responses upstream of classical lymphoid priming of naïve T cells (Fonseca et al., 2020).

1.2 Inflammation as a driver of hematopoietic and immune remodeling

Inflammation is a protective tissue response that is triggered when infection, injury, or other forms of stress disturb homeostasis. Importantly, it includes both **pro-inflammatory** programs that contain danger and activate effector mechanisms, and **anti-inflammatory** or pro-resolving programs that limit collateral damage, support the clearance of dying cells, and promote restoration of tissue integrity. The biological outcome therefore depends not only on the magnitude of inflammatory activation, but also on its timing, composition, and resolution (Cicchese et al., 2018). Following antigen recognition, proximal **signaling cascades** relay the stimulus into distal signaling programs that converge on inflammatory transcription factors. Although many of the core signaling modules are conserved across cell types, their effects on functional **polarization** are highly context dependent. Through the production of **cytokines** and **chemokines**, local sensing events are propagated within the tissue microenvironment and to the systemic level. Together, these signals instruct cell identity, drive tissue remodeling, and hematopoietic lineage output.

1.2.1 Principal signals of inflammation

The receptor families that initiate inflammatory responses are germline-encoded **pattern recognition receptors** (PRRs) of the innate immune system (R. Chen et al., 2025; Metschnikoff, 1891) and **antigen-specific receptors** on B cells (the B cell receptor or BCR) and T cells (the T cell receptor or TCR) of the lymphoid immune system (Ehrlich, 1900; Shi et al., 2024). In the following century the two branches were explored largely in parallel until the discovery of innate antigen presenting cells (APCs), which established a mechanistic link between innate sensing and adaptive immune activation (Paul, 2011). In the current framework, PRR activation in innate sentinel cells promotes antigen-specific lymphoid responses through at least three interconnected functions: it enhances antigen processing and presentation in the context of MHC molecules, induces co-stimulatory ligands on antigen-presenting cells, and drives the production of early instructive cytokines that shape lymphocyte activation and differentiation. Activated lymphocytes then produce downstream effector cytokines that amplify or resolve inflammation, modulate myeloid-cell activation and interferon programs, and indirectly support tissue repair processes, including the phagocytic clearance of apoptotic cells (Carroll et al., 2024). This classical division has since become less rigid. PRRs are now known to be expressed not only by innate immune cells, but also by lymphoid (Bourke et al., 2003; Caramalho et al., 2003) and non-hematopoietic cells (Miettinen et al., 2001).

Antigen specific receptor families

B cell receptor (BCR) and T cell receptor (TCR) genes are generated by RAG1- and RAG2-mediated somatic recombination of germline variable (V), diversity (D), and joining (J) gene segments, followed by transcription and splicing to constant-region exons (Schatz & Ji, 2011). Their variable domains contain three complementarity-determining regions (CDR1–3). Whereas CDR1 and CDR2 are encoded within the germline V gene segment, CDR3 is formed at the VJ or VDJ junction and represents the most diverse determinant of antigen specificity (Schatz & Ji, 2011). In B cells, the **membrane-bound BCR** consists of two identical VJ-recombined light chains and two identical VDJ-recombined heavy chains. The heavy chain is upstream to an isotype-defining constant region gene, such that mature naïve B cells typically co-express IgM and IgD through alternative splicing of the same rearranged heavy-chain transcript, whereas activated B cells can undergo class-switch recombination to express IgG, IgA, or IgE (Dong et al., 2022; J.-C. Liu et al., 2024). The same rearranged immunoglobulin gene can generate either a membrane-bound BCR or a **soluble antibody** through alternative RNA processing. In naïve and mature B cells, immunoglobulin is expressed primarily as a membrane-bound BCR, whereas antigen-driven activation usually induces differentiation into plasmablasts and plasma cells that secrete antibody, although innate-like B-1 cells can constitutively produce natural IgM even in the absence of foreign antigen exposure (Kaveri et al., 2012). **Membrane-bound TCR** chains are encoded by the TRA, TRB, TRG, and TRD loci and assemble into either $\alpha\beta$ or $\gamma\delta$ heterodimers (Shah et al., 2021). The α chain is generated by VJ recombination, whereas the β chain is generated by VDJ recombination and subsequently spliced to the constant regions TRAC and TRBC, respectively. In contrast to conventional $\alpha\beta$ T cells, $\gamma\delta$ T cells often display restricted or semi-invariant receptor usage and can recognize antigens independently of classical peptide–MHC presentation, although the $\gamma\delta$ T cell compartment is heterogeneous and should not be considered uniformly invariant (Hu et al., 2023). By contrast, innate-like $\alpha\beta$ T cells such as mucosal-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells express semi-invariant rather than truly invariant TCRs (Garner et al., 2018). Antigen recognition by both the B cell receptor (BCR) and T cell receptor (TCR) triggers signaling through immunoreceptor tyrosine-based activation motifs (ITAMs). In B cells, these motifs are provided by the receptor-associated subunits Ig α (CD79A) and Ig β (CD79B), whereas the $\alpha\beta$ TCR signals through the invariant CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$ and $\zeta\zeta$ dimers. Conventional $\alpha\beta$ T cells recognize antigen only as peptide bound to MHC molecules, with CD4 or CD8 acting as co-receptors that support receptor signaling. By contrast, the BCR recognizes intact native antigen in an MHC-independent manner. Nevertheless, productive activation of B cells in T-dependent immune responses generally requires cognate CD4⁺ T cell help following antigen uptake and presentation on MHC class II. In B cells, BCR action supports antigen uptake and presentation, survival, and differentiation into germinal-center,

memory, or antibody secreting plasma cells. TCR activation can support activation, proliferation, and differentiation into diverse functional states, including effector, memory, tolerance, or exhausted programs (Shah et al., 2021). In both lineages, however, the exact biological output is highly context dependent.

Pattern recognition receptor families

Pattern recognition receptors are a diverse group of germline encoded receptors that sense various **pathogen associated molecular patterns** (PAMPs) (Janeway & Medzhitov, 2002; Medzhitov, 2009). Later it became clear that they also sense **damage associated molecular patterns** (DAMPs) released from damaged or apoptotic cells (Matzinger, 1994). Given their role in maintaining tissue homeostasis and immune tolerance toward commensal microorganisms, multiple constitutive immune mechanisms (Paludan et al., 2021) and inhibitory PRRs (Rumpret et al., 2022) operate to suppress inappropriate inflammatory responses. Cellular PRRs exist as either membrane-bound or cytosolic entities and are specialized to detect defined classes of conserved molecular patterns (Takeuchi & Akira, 2010).

Membrane-bound PRRs include C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). CLRs belong to the C-type lectin superfamily (Zelensky & Gready, 2005) and comprise a versatile group of receptors that regulate phagocytosis, cytokine production, and antigen presentation (Reis e Sousa et al., 2024). They are expressed predominantly by myeloid cells and often display cell type- or activation state-specific expression patterns, exemplified by mannose receptor (MRC1) on alternatively activated macrophages, Langerin (CD207) highly expressed on Langerhans cells, and DNGR-1 (CLEC9A), a defining marker of type 1 conventional DCs (Reis e Sousa et al., 2024). Toll-like receptors were the first mammalian PRRs to be molecularly characterized (Fitzgerald & Kagan, 2020). Humans express 10 TLRs (TLR1-TLR10), whereas mice express 12 functional TLRs (TLR1-TLR9 and TLR11-TLR13). Cell-surface TLRs, including TLR1, TLR2, TLR4, TLR5, TLR6, and human TLR10, primarily detect microbial membrane components such as lipids, lipoproteins, or flagellin. Endosomal TLRs, including TLR3, TLR7, TLR8, and TLR9, specialize in sensing nucleic acids such as double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and unmethylated cytosine-phosphate-guanine DNA (CpG DNA) motifs. TLRs are type I transmembrane receptors with a conserved cytoplasmic Toll/IL-1 receptor (TIR) domain that signal through TIRAP–MyD88 or TRAM–TRIF (Kawai et al., 2024). Notably, TLRs are widely expressed in both hematopoietic and non-hematopoietic cells.

Cytosolic PRRs include retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase (cGAS), absent in melanoma 2 (AIM2)-like receptors (ALRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Takeuchi & Akira, 2010). **RLRs** such as RIG-I and MDA5

detect viral or aberrantly localized RNA in the cytosol and signal through the mitochondrial adaptor MAVS to induce antiviral and inflammatory transcriptional programs (Rehwinkel & Gack, 2020). Similarly, **cGAS** functions as a cytosolic double-stranded DNA (dsDNA) sensor that catalyzes the production of cyclic GMP-AMP, thereby activating STING (Z. Zhang & Zhang, 2025). Together, RLRs and cGAS-STING signaling constitute the principal cytosolic pathways that couple nucleic acid sensing to antiviral immunity and IFN-I production. **ALRs** constitute another family of cytosolic dsDNA sensors but primarily link DNA recognition to **inflammasome** activation, most prominently through AIM2 (Kumari et al., 2020; Martinon et al., 2002). The **NLR** family is divided by N-terminal effector-domain architecture into NLRA, NLRB, NLRC and NLRP subfamilies (Kanneganti et al., 2007). Most NLRs share a central NACHT domain mediating oligomerization and C-terminal LRR motifs involved in ligand sensing and autoregulation. Functionally, NLRs are heterogeneous. NLRA regulates MHC class II transcription, NLRC1 (NOD1) and NLRC2 (NOD2) activate NF- κ B/MAPK signaling, and selected NLRs such as NLRP1, NLRP3 and NLRC4 assemble inflammasomes.

Inflammatory signaling pathways and cytokine-mediated crosstalk

Antigen binding activates a set of cooperative signaling hubs (Cao, 2016) that induce transcriptional programs by activation of the transcription factors NF- κ B (Q. Guo et al., 2024), AP-1 (Bejjani et al., 2019), and IRFs (Lian Wang et al., 2024). Activation of NF- κ B and AP-1 drives the expression of various cytokines such as TNF and IL-6 that are rapidly induced and released, allowing them to exert pleiotropic effects on immune and non-immune cells (Murakami et al., 2019). In contrast, IL-1 β and IL-18 are regulated by an additional post-translational checkpoint. These cytokines are synthesized as inactive cytosolic precursors, pro-IL-1 β and pro-IL-18, which are not efficiently secreted through conventional pathways (Ford et al., 2025). Their maturation depends on inflammasome activation by assembly of its respective canonical sensory components (AIM2, pyrin, NLRP1, NLRP3, or NLRC4) which connect to caspase 1 via the adaptor protein ASC (*PYCARD*) (Broz & Dixit, 2016). Upon inflammasome activation caspase-1 cleaves pro-IL-1 β and pro-IL-18 into mature IL-1 β and IL-18. Active caspase-1 also cleaves gasdermin D, releasing its pore-forming N-terminal fragment, which promotes cytokine release and pyroptotic cell death (Broz, 2025). Importantly, inflammasome activation depends on two signals (Latz et al., 2013). The first signal primes inflammasome pathways through the expression and posttranslational modification of its components and substrate precursors. This stage can be induced by various PAMPs, DAMPs, and inflammatory cytokines. The second signal is generally associated with severe cellular danger and ultimately leads to assembly and activation of the respective inflammasome. While the AIM2 inflammasome is directly activated by dsDNA, the NLRC4 inflammasome is activated by the cytosolic upstream sensor NAIP, which senses conserved bacterial virulence structures (Kofoed & Vance, 2011). NLRP3 is unique in that it can be activated by a wide range of

unrelated stimuli that induce cellular stress (Swanson et al., 2019). This includes lysosomal disruption, efflux of potassium or chloride ions, calcium ion flux, mitochondrial dysfunction, and metabolic changes. However, how these pathways integrate and overlap with other inflammatory signals is not fully understood.

Among the interferon regulatory factor (IRF) family, IRF3 and IRF7 are central transcriptional regulators of IFN-I induction (Boehmer & Zanoni, 2025). In contrast to IFN-II, which is represented by the single cytokine IFN- γ , IFN-I and IFN-III comprise multigene cytokine families. In humans, the IFN-I family includes 13 IFN- α subtypes together with IFN- β , IFN- ϵ , IFN- κ and IFN- ω , whereas mice encode 14 IFN- α subtypes together with IFN- β , IFN- ϵ , IFN- κ and IFN- ζ . Type III interferons comprise the IFN- λ family, with humans encoding four (IFN- λ 1, IFN- λ 2, IFN- λ 3 and IFN- λ 4), while mice encode two (IFN- λ 2 and IFN- λ 3) subtypes (Platanias, 2005). Upon activation of nucleic acid-sensing pathways, including endosomal TLRs, RIG-I-like receptors, and the cGAS-STING axis, IRF3 and IRF7 cooperate with NF- κ B and AP-1 to induce IFN-I expression. Secreted IFN-I signals through the heterodimeric interferon- α/β receptor (IFNAR), activating JAK1 and TYK2 and thereby promoting STAT1 and STAT2 phosphorylation (Lazear et al., 2019). STAT1 and STAT2 subsequently associate with IRF9 to form interferon-stimulated gene factor 3 (ISGF3), the canonical transcriptional complex that binds interferon-stimulated response elements (ISREs) and drives interferon-stimulated gene (ISG) expression. IFN-I was initially discovered as mediators of viral interference (Isaacs & Lindenmann, 1957), but IFN-I induced ISG programs are now recognized to extend far beyond antiviral restriction, regulating antibacterial defense, cytokine signaling, antigen presentation, cell survival, and immune-cell activation (González-Navajas et al., 2012). Although IFN-I and IFN-II activate partially overlapping ISGs, their effects on macrophage activation diverge substantially in response to virus, bacteria, parasites, and fungi (McNab et al., 2015). Whereas IFN-II acts primarily as an effector cytokine that promotes classical antimicrobial macrophage activation, IFN-I more broadly modulates macrophage polarization and reprograms metabolic functions, including iron homeostasis and efferocytosis (Adler & Adler, 2022; Dunphy et al., 2026; Riedelberger et al., 2020). In addition to these inducible functions, IFN-I also contributes to immune homeostasis through low-level tonic signaling, which maintains basal ISG expression and tunes immune-cell responsiveness at steady state (Gough et al., 2012). However, when IFN-I signaling is excessive or sustained, the same pathway can drive interferonopathies and contribute to chronic inflammation and autoimmunity (Boehmer & Zanoni, 2025; Schoggins, 2019).

1.2.2 Macrophage and T cell activation as context-dependent state transition

The sensing of inflammatory mediators has profound effects on immune cell state and function. Early classification systems divided immune cells into discrete categories according to their effector profiles (C. D. Mills et al., 2000). While these schemes are valuable for establishing

broad functional principles, they do not fully reflect the biological complexity of immune responses *in vivo* (Masopust et al., 2026; Murray et al., 2014). The concept of **polarization** arose from the recognition that immune cell behavior is highly context dependent and often reversible, rather than representing a discrete and deterministic terminal state (Mantovani et al., 2012). Complementing this view, the concept of immune memory highlights that immune responses are also shaped by prior experience, such that the functional state of a cell reflects not only the immediate stimulus but also the cumulative imprint of previous perturbations (Hajishengallis et al., 2023; N. Lam et al., 2024; Zahalka et al., 2022).

The complex history of macrophage terminology is rooted in the observation that macrophages can adopt both inflammatory and homeostatic phenotypes (Nathan et al., 1983; Stein et al., 1992). In 2000 macrophages were first formally categorized into M1 and M2 states based on their differences in metabolism and function (C. D. Mills et al., 2000). In the current frameworks (Murray et al., 2014), M1 macrophages are generated by stimulation with TLR agonist or inflammatory cytokines IFN- γ and TNF- α . M1 macrophages are defined by elevated production of pro-inflammatory mediators (IL-1 β , IL-6, IL-12, and TNF- α), together with secretion of nitric oxide (NO), reflecting a functional state specialized for antimicrobial defense and inflammatory amplification. In contrast, M2 macrophages were subdivided into several states based on their activation stimulus (M2a: IL-4 and IL-13, M2b: Immune complexes, TLR ligands and IL-1b, M2c: IL-10, TGF- β , and Glucocorticoids and M2d: IL-6, TLR ligands, and Adenosine) (Röszer, 2015). M2 are characterized by increased phagocytic capacity and production of anti-inflammatory cytokines (IL-10 and TGF- β), chemokines (CCL17, CCL18, CCL22, CCL24), growth factors and angiogenic mediators (TGF- β , VEGF, PDGF, EGF, BMP-2, IGF) that generally support immunoregulation, resolution of inflammation, tissue remodeling (Bosco, 2019; Shapouri-Moghaddam et al., 2018). *In vivo* evidence for macrophage repolarization has been provided in sterile injury models, where recruited inflammatory monocytes transition locally toward anti-inflammatory or reparative states during tissue repair (Arnold et al., 2007; Dal-Secco et al., 2015). In the first manuscript presented in this thesis (Chapter 2.1), we investigate how splenic red pulp macrophages transition across inflammatory activation states to maintain iron homeostasis.

Conventional dendritic cells type 1 (cDC1) and type 2 (cDC2) are the principal antigen-presenting cells for naïve T cell priming (Eisenbarth, 2019). cDC1 are particularly efficient at cross-presentation on MHC class I, whereas cDC2 are more commonly associated with MHC class II-dependent priming of CD4⁺ T cells. Following antigen encounter, activated DCs acquire a CCR7-dependent migratory program and traffic from peripheral tissues through afferent lymphatics to draining lymph nodes or, in the spleen, reposition from the red pulp or marginal zone to the white pulp, where they colocalize with naïve T cells (Ato et al., 2002; Calabro et al., 2016). The activation of naïve T cells occurs in at least three distinct stages

(Mempel et al., 2004), and current evidence indicates that microanatomical positioning within the lymph node T cell zone or the periarteriolar lymphoid sheath of the splenic white pulp is an important determinant of subsequent T cell differentiation (Eisenbarth, 2019; S.-W. Lee et al., 2023). Depending on the cytokine milieu and co-stimulatory context, activated T cells induce distinct transcriptional programs that bias differentiation toward cytotoxic CD8⁺ T cells or toward CD4⁺ helper T cell states, including T_{H1} (T-bet), T_{H2} (GATA3), T_{H17} (RORγt), T_{reg} (FOXP3) and T_{FH} (BCL6) (D. Zou et al., 2025). CD4⁺ T_{H1} cells produce IFN-γ, which promotes M1 macrophage programs, whereas CD4⁺ T_{H2} cells produce IL-4 and IL-13, which favor M2 activation. By contrast, CD4⁺ T_{H17} cells support barrier protection by producing IL-17A, IL-17F, and IL-22, which act mainly on epithelial and stromal cells to promote antimicrobial defense and neutrophil recruitment (K. H. G. Mills, 2023). T_{reg} primarily restrain immune activation and maintain peripheral tolerance through suppressive mechanisms including IL-10 and TGF-β production, while T_{FH} cells produce IL-21 and provide B cell help rather than serving as major direct regulators of macrophage polarization (Sun et al., 2023). Traditionally, CD8⁺ T cells have been characterized by their ability to release cytotoxic granules to kill target cells (Zinkernagel & Doherty, 1974). It was later shown that the CD8⁺ T cell subsets T_{C1} and T_{C2} can produce effector cytokines analogous to those of their CD4⁺ T cell counterparts (Sad et al., 1995). Similar to macrophage polarization states, CD8⁺ T cell polarization states have been linked to pathological conditions, although the functional significance of some subsets remains debated (Koh et al., 2023). However, in mice, distinct skin-resident CD8⁺ T differentiation pathways have been identified. While CD8⁺ T_{1DRM} depend on a T-bet–Hobit–IL-15 axis, CD8⁺ T_{17DRM} subsets are established by an ICOS–c-Maf–IL-7 pathway (Simone L. Park et al., 2023). As for macrophages, T cell polarization is increasingly understood as a dynamic and tissue-instructed process, in which local cytokines, antigen persistence, and metabolic cues can reshape transcriptional and functional states during and after initial differentiation (Guéry & Hugues, 2015; Karmaus et al., 2019; Zhu & Paul, 2010). In the second manuscript (Chapter 2.2), we investigate skin T_{DRM} adaptation and retrograde migration after HSCT in the human skin.

1.2.3 Effects of inflammation on hematopoiesis and immune output

During inflammation, hematopoiesis rapidly adapts to meet the increased demand for myeloid effector cells, a process termed **emergency myelopoiesis** (Swann et al., 2024). Whereas myeloid output is increased, lymphoid and erythroid development is concomitantly suppressed (Johnson et al., 1991; Libregts et al., 2011; Ueda et al., 2004). Inflammatory remodeling occurs across the hematopoietic hierarchy. IFN-I and IFN-II activate HSC and IL-1 enforces PU.1 expression which preferentially produces myeloid-biased MPP2 and MPP3 populations (Baldridge et al., 2010; Essers et al., 2009; Pietras et al., 2016; Sato et al., 2009). Forced

activation of HSC can permanently impair self-renewal capacity and is related to aging (Bogeska et al., 2022; Caiado et al., 2021). However, inflammatory stress can durably reprogram HSCs and MPPs at the epigenetic level, generating a memory-like hematopoietic state that contributes to **central trained immunity** (Netea et al., 2020). Fate-mapping and progenitor-transfer studies proposed that, in LPS-challenged mice, bone marrow GMPs preferentially generate neutrophil-like Ly6C^{hi} monocytes, whereas CpG challenge favors MDP-derived DC-like Ly6C^{hi} monocytes (Yáñez et al., 2017). Therefore, inflammatory stress alters not only the quantity but also the qualitative properties of cell output from the bone marrow. Inflammation not only affects the bone marrow but also sites of adult **extramedullary hematopoiesis**. While hematopoietic stem and progenitor cells (HSPC) can be found in several adult tissues, extramedullary hematopoiesis is most prominent in the spleen and liver (Mende & Laurenti, 2021). In mice, this process can be induced under both homeostatic and pathological conditions, whereas in humans it is observed predominantly in the context of disease. During inflammation, the spleen supports both granulopoiesis and **stress erythropoiesis**, thereby buffering reduced bone-marrow output (Bennett et al., 2018; Johns & Christopher, 2012). The first manuscript presented in this thesis (Section 2.1) investigates how inflammatory signals that suppress bone marrow erythropoiesis are integrated within the splenic erythroid niche to support stress erythropoiesis in mice.

The following chapters examine two distinct tissue environments, the skin as a barrier organ and the spleen as a systemic immune niche. These complementary systems provide a framework to investigate how inflammation shapes immune-cell polarization and tissue residency.

1.3 The spleen as a systemic immune niche

The spleen is a highly organized secondary lymphoid organ that is structurally and functionally divided into distinct anatomical compartments, each supporting specialized aspects of immune surveillance of blood-borne pathogens and antigens (Lewis et al., 2019; Mebius & Kraal, 2005). Beyond its role as a filter of the circulation, the spleen functions as a site of extramedullary hematopoiesis (Johns & Christopher, 2012) and contributes to systemic homeostasis, most notably through the regulation of iron metabolism (Vogt et al., 2021). It is important to note that several of the aforementioned functions are also performed by the liver, although a detailed comparison of these organs is beyond the scope of this thesis (Cenariu et al., 2021). In the following sections, this thesis first outlines the anatomical organization of the spleen, including a brief description of the mechanisms enabling rapid initiation of adaptive immune responses. This is followed by a discussion of the ontogeny and function of splenic tissue-resident macrophages, with particular emphasis on red pulp macrophages and their role in extramedullary erythropoiesis.

1.3.1 The anatomy and function of the spleen as a systemic immune niche

At the macroscopic level, the spleen is composed of red pulp and white pulp which are interspersed and connected by a complex vascular network. The main anatomical compartments and spatial organization of the spleen are schematically depicted in Figure 3.

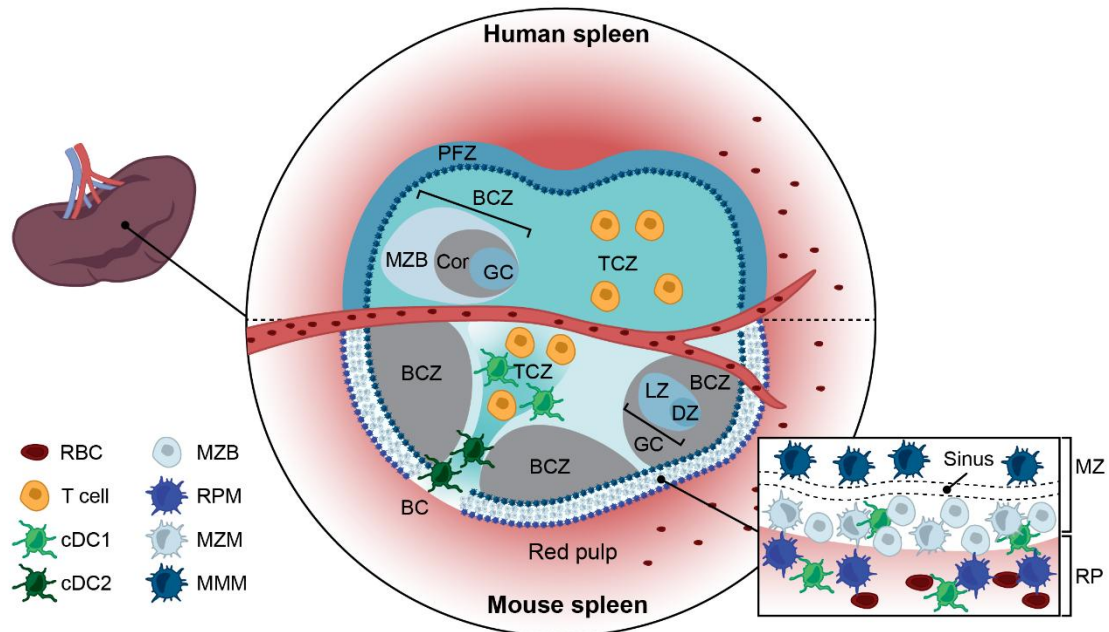


Figure 3 Anatomical organization of the human and mouse spleen.

Schematic illustration of splenic architecture in humans and mice. The upper part depicts the anatomical organization of the human spleen, whereas the lower part illustrates the mouse spleen. The inset in the lower right shows the immune-cell organization of the mouse marginal zone. Abbreviations: BC, bridging channel; BCZ, B cell zone; cDC, conventional dendritic cell; Cor, corona; DZ, dark zone; GC, germinal center; LZ, light zone; MMM, marginal metallophilic macrophage; MZ, marginal zone; MZB, marginal zone B cell; MZM, marginal zone macrophage; PFZ, perifollicular zone; RBC, red blood cell; RP, red pulp; RPM, red pulp macrophage; TCZ, T cell zone.

The **white pulp** is organized around central arteries and forms discrete lymphoid structures that resemble lymph nodes. Naïve T and B cells continuously circulate through the spleen, where antigen-encounter within the white pulp drives their activation and differentiation into effector T cells and antibody-secreting plasma cells. Segregation of T and B cell compartments in the splenic white pulp is primarily orchestrated by stromal cell-derived chemokines, with CCL19 and CCL21 directing CCR7-expressing T cells and DCs to the periarteriolar lymphoid sheath, and CXCL13 positioning CXCR5-expressing B cells within follicles (Lewis et al., 2019). Splenic cDC1 (XCR1⁺CD8α⁺) are efficiently cross-presenting blood-borne antigens to naïve CD8⁺ cytotoxic T cells and while cDC2 (SIRPα⁺CD11b⁺) present antigen via MHC II to naïve CD4⁺ helper T cells. T follicular helper cells (CCR7⁻ CXCR5⁺) migrate to the B cell follicles where they provide essential signals (IL-21, CD40L and ICOS) to antigen-experienced B cells to promote survival, germinal center formation, and immunoglobulin class switching (Dave & Ballesteros-Tato, 2025). An important anatomical distinction of the spleen in comparison to

lymph node is the absence of a capsule separating white pulp from parenchyma of the spleen (red pulp). Instead, the **marginal zone (MZ)** acts as an interface between circulation and lymphoid tissue, containing specialized macrophages and marginal zone B cells positioned to capture and present antigens (Pillai & Cariappa, 2009). In contrast, the **red pulp** constitutes most of the splenic tissue and is characterized by a network of splenic cords and venous sinusoids. This compartment is optimized for the filtration of blood, facilitating the clearance of circulating pathogens and the removal of senescent erythrocytes. The open vascular architecture of the red pulp imposes mechanical constraints on circulating cells, enforcing prolonged transit times and enabling extensive interactions between blood cells and resident macrophages.

1.3.2 Tissue-resident macrophages of the spleen

Blood-borne antigens are first encountered in the marginal zone, where specialized tissue resident macrophages mediate their capture and can interact with the adaptive immune system. The two principal subsets are **marginal zone macrophages (MZMs)** and **marginal metallophilic macrophages (MMMs)**, retained by CCL21 chemokine signals (Mebius & Kraal, 2005). Both populations are generally regarded as tissue resident macrophages of predominantly embryonic origin and display a broad repertoire of PRRs that detect both PAMPs and DAMPs. **MZMs** reside in the outer marginal zone and are defined by expression of the scavenger receptor MARCO and the lectin receptor SIGN-R1 (CD209b). Notably, expression of SIGN-R1 on MZMs is partly regulated by marginal zone B cells (You et al., 2011). They mediate clearance of apoptotic cells and blood-borne pathogens by recognizing pathogen-associated polysaccharides as well as antibody- and complement component C1q (C1q) opsonized antigens (Koppel et al., 2008). While they do not seem to initiate adaptive immune responses, they are important for response maturation and the formation of germinal center B cells (Pirgova et al., 2020). Depletion of MZMs results in elevated proinflammatory cytokine levels during infection and accelerates autoimmune disease progression in mice (McGaha et al., 2011). **MMMs** line the inner border of the marginal zone adjacent to the white pulp and are defined by high expression of CD169 (Siglec-1). Compared to MZMs, they exhibit lower phagocytic activity but do support antigen transport to the white pulp and can cross-present antigens to CD8⁺ cytotoxic T cells (Mauvais et al., 2025). An interesting feature of MMMs is that they selectively permit transient controlled viral replication within themselves. This enforced viral replication enables robust adaptive immune priming (Honke et al., 2011). Germinal centers support rapid proliferation and selection of B cells during adaptive immune response. This leads to accumulation of apoptotic B cells which failed proper B cell receptor affinity maturation or did not receive T cell help. **Tingible body macrophages (TBMs)**, which reside in the germinal center, efficiently remove apoptotic B cells and associated self-reactive

debris, thereby preventing B cell mediated autoimmune disease (Grootveld et al., 2023). **Red pulp macrophages** (RPMs) are localized within the splenic red pulp, where they are strategically positioned along the sinusoidal vasculature to continuously survey the blood. In contrast to other tissue-resident macrophage populations, including alveolar, peritoneal, and bone marrow macrophages, RPMs show minimal bromodeoxyuridine incorporation, indicating very limited proliferative activity at steady state. However, after non-genotoxic depletion, the remaining RPMs can proliferate and locally restore the compartment, supporting the idea that RPMs retain self-renewal capacity (Hashimoto et al., 2013). Their primary function is the clearance of senescent or damaged erythrocytes, a process that is tightly linked to systemic iron homeostasis (Hentze et al., 2004). RPMs recognize aged red blood cells through alterations in membrane composition and deformability, leading to phagocytic uptake and intracellular degradation of hemoglobin. In addition, RPMs contribute to erythropoiesis by forming **erythroblastic islands**, specialized niches in which they interact with erythroid precursors and support their proliferation and maturation, functionally analogous to nursing macrophages in the bone marrow (Klei et al., 2017). A central determinant of RPM development is the transcription factor SPI-C. It is highly expressed in RPMs and is required for efficient erythrophagocytosis and iron metabolism (Halder et al., 2014; Hentze et al., 2004). Heme is an erythroid metabolite which is released during erythroid degradation and SPI-C expression is induced by heme dependent degradation of its transcriptional suppressor BACH1. Interestingly, heme dependent activation of SPI-C is sufficient for monocyte and bone marrow macrophages to adopt a transient RPM phenotype (Halder et al., 2014). Recent work has shown that further maturation of monocyte derived RPMs depends on IL-33 signaling and MyD88 activation as a response to physiological erythrocyte damage (Lu et al., 2020). Although RPMs express Toll-like receptors such as TLR4 and TLR9, activation of these pathways does not appear to induce a strong classical pro-inflammatory cytokine program. Instead, TLR4 or TLR9 mediated stimulation has been associated primarily with production of the anti-inflammatory mediators IL-10 and TGF- β , as well as with induction of regulatory T cell responses (Kurotaki et al., 2011).

1.3.3 Red pulp macrophages as regulators of erythroid homeostasis

In adult mice, the spleen contributes to steady-state extramedullary erythropoiesis and provides a specialized microenvironment in which erythroid maturation and iron recycling are closely coupled (Klei et al., 2017). In humans, by contrast, steady-state erythropoiesis is predominantly restricted to bone marrow, and the spleen usually becomes haematopoietically relevant only during fetal life or under pathological conditions. This species difference is important, because the murine splenic red pulp represents a physiologically active erythroid niche (Mende et al., 2022). **Erythropoiesis** proceeds from megakaryocyte-erythroid

progenitors through burst-forming (BFU-E) and colony-forming (CFU-E) erythroid progenitors toward proerythroblasts, basophilic, polychromatic and orthochromatic erythroblasts, before reticulocyte release (K. Chen et al., 2009). This process is schematically shown in Figure 4 and is coordinated by stage-specific signaling pathways and transcriptional programs (S.-I. Kim & Bresnick, 2007). Early progenitors depend on SCF–c-KIT signaling to preserve survival and proliferative capacity, whereas CFU-E and early erythroblasts become increasingly dependent on EPO–EPOR signaling (Țichil et al., 2024). At the transcriptional level, erythroid commitment and maturation are driven by GATA1, KLF1 and TAL1, while terminal differentiation is reinforced by regulators such as SOX6 (Cha, 2024).

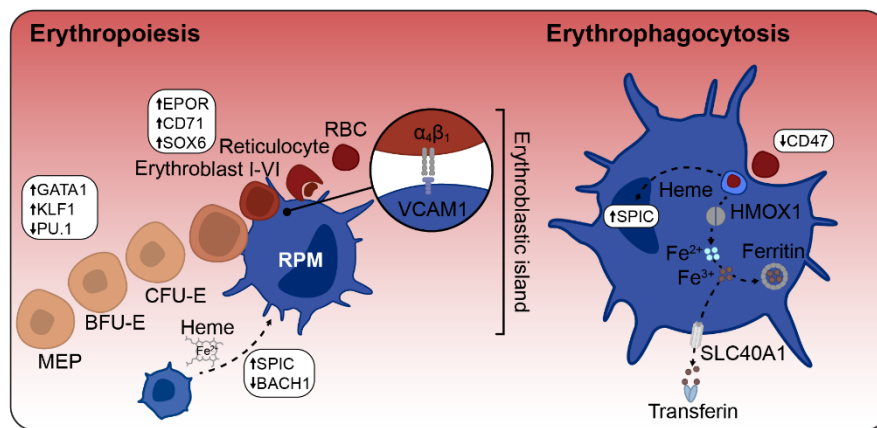


Figure 4 Red pulp macrophage coordinate erythropoiesis and iron homeostasis.

The left side illustrates erythroid development, including key transcription factors and the organization of splenic erythroblastic islands, with RPMs functioning as nursing macrophages. The lower panel summarizes principles of monocyte differentiation into RPMs. The right side depicts erythrophagocytosis and the basic iron-handling pathway of RPMs. Abbreviations: BFU-E, burst-forming unit erythroid; CFU-E, colony-forming unit erythroid; HMOX1, heme oxygenase 1; MEP, megakaryocyte–erythroid progenitor; RBC, red blood cell; RPM, red pulp macrophage; SLC40A1, ferroportin; VCAM1, vascular cell adhesion molecule 1.

As erythroblasts mature, they progressively upregulate transferrin receptor 1 (CD71) to maximize iron uptake to be incorporated into hemoglobin. Within erythroblastic islands, macrophages stabilize erythroblast interactions through adhesion pathways including VCAM1– $\alpha_4\beta_1$ integrin and express molecules such as CD169, VCAM1, and MERTK (Fang et al., 2025). In addition, red pulp macrophages mediate the clearance of extruded nuclei and mitochondria during terminal erythroid maturation (Qiu et al., 1995). A central function of splenic red pulp macrophages is the **recycling of iron** from senescent erythrocytes (Figure 4). During erythrophagocytosis, engulfed senescent erythrocytes are degraded in the phagolysosome, where hemoglobin is dismantled and heme is released. A central step in this process is its catabolism by heme oxygenase 1 (HMOX1), which cleaves heme to generate biliverdin, carbon monoxide, and ferrous iron (de Oliveira et al., 2022). The liberated iron then enters a tightly controlled intracellular trafficking network. Cytosolic iron can be transiently buffered in ferritin, thereby limiting redox toxicity, or directed toward export through ferroportin (SLC40A1), the only known cellular iron exporter, enabling its loading onto circulating

transferrin for redistribution to erythroid precursors (Roemhild et al., 2021). Upstream of this, heme derived from phagocytosed erythrocytes is transported from the phagolysosome compartment into the cytosol by the heme transporter SLC48A1, thereby coupling erythrophagocytosis to intracellular heme processing. Because most iron required for ongoing erythropoiesis is supplied by recycling rather than intestinal absorption alone, red pulp macrophages are integral to systemic iron homeostasis. Their role is therefore not limited to clearance of aged erythrocytes but extends to sustaining continuous hemoglobin synthesis in developing erythroblasts.

1.4 The skin as a local immune barrier

Did terrestrial vertebrates ever truly leave the ocean, or do they carry an ocean within? From an evolutionary perspective, the emergence of skin adapted to retain water was the most important adaptation that enabled fish to transition onto land. However, beyond its role in preventing desiccation, the skin functions as a complex barrier organ that orchestrates a broad spectrum of interactions between the organism and its external environment. This includes regulation of water retention, sensory perception, thermoregulation, shielding against UV radiation, and importantly, immune defense (Baker et al., 2023). In the following, this thesis considers the skin primarily as an **immunological barrier organ** and examines the diverse repertoire of tissue-resident immune cells that shape local homeostasis, immune surveillance, and inflammatory remodeling.

1.4.1 The anatomy and function of the skin as an immunological barrier organ

The skin is organized into three distinctive layers (Zareie et al., 2025). The **epidermis** constitutes the outermost layer of the skin and is composed predominantly of keratinocytes, which account for approximately 90–95% of its cellular content (Simmons & Gallo, 2024). Upon differentiation and outward migration, keratinocytes form the **stratum corneum**, where terminally differentiated dead cells provide a physical and chemical barrier that restricts pathogen entry and reduces water loss (Elias, 2005). Keratinocytes build a complex network with the microorganisms of the skin, express PRRs (e.g., TLR) and can produce a wide range of cytokines (e.g., TNF, IL-1, IL-6, IFN- β , IFN- γ), chemokines (e.g., CXCL2, CXCL8, CCL20), antimicrobial peptides and promote regulatory T cell function and homeostasis by releasing immunomodulatory cytokines (e.g., TGF- β) (Gallo & Hooper, 2012; Simmons & Gallo, 2024). Similarly, neural crest-derived melanocytes not only produce melanin to protect against ultraviolet radiation but also act as sentinel cells with immunological functions (Centeno et al., 2023). Most skin microorganisms do not primarily reside on the exposed stratum corneum but preferentially inhabit adnexal skin structures, niches such as hair follicles and sweat or sebaceous glands. Commensal communities, including *Staphylococcus epidermidis*, *Cutibacterium acnes*, and *Malassezia* species, actively contribute to cutaneous immune

homeostasis and defense (Nakatsuji et al., 2021). In human skin, the epidermis contains tissue-resident CD4⁺ and CD8⁺ T cells, $\gamma\delta$ T cells, and Langerhans cells, which together support local immune surveillance (Zareie et al., 2025). In mice, the epidermis additionally harbors dendritic epidermal T cells (DETCs), a specialized $\gamma\delta$ T cell population characterized by an invariant V γ 5V δ 1 T cell receptor repertoire. Langerhans cells are the embryonic derived tissue-resident macrophages of the skin, but were initially categorized as DCs because of their specialization in antigen presentation and their capacity to enter the lymphatic system to prime naïve T cells (Doebel et al., 2017). Together with keratinocyte-derived signals, they communicate danger signals to deeper layers of the skin and thereby initiate local and adaptive immune responses. The middle layer of the skin is the **dermis**. Its stroma comprises among others endothelial cells that form the vascular networks linking the skin to the circulation and fibroblasts. Skin fibroblasts exhibit remarkable plasticity and, similar to keratinocytes, can produce a wide range of immune mediators (Steele et al., 2025). By producing and remodeling extracellular matrix components, they help maintain tissue architecture and support wound healing (Plikus et al., 2021). Under homeostatic condition the dermis hosts a wide range of innate immune cells including bone marrow derived DCs (cDC1 and cDC2) (Kashem et al., 2017), tissue-resident macrophages (S. H. Lee & Sacks, 2024), mast cells (Voss et al., 2021) and innate lymphoid cells (ILC1, ILC2, ILC3, NK cells and LTi) (Vivier et al., 2018). ILC1, ILC2 and ILC3 produce similar cytokine profiles to their corresponding CD4⁺ subsets T_{H1}, T_{H2}, and T_{H17}. In healthy human skin, ILC2s and ILC3s constitute the predominant innate lymphoid cell populations maintaining homeostasis and modulate defense against extracellular parasites, bacteria or fungi (Gladiator et al., 2013; Roediger et al., 2013). ILC1 and NK cells infiltrate inflamed skin in response to intracellular pathogens but can adapt tissue residency (Torcellan et al., 2024; Weizman et al., 2017). While the epidermis is enriched for CD8⁺ T cells that localize to sites of previous infection, the dermis contains most CD4⁺ T cells, which also display greater motility. (Gebhardt et al., 2011). Figure 5 schematically shows the principal anatomical organization of the epidermis and dermis, together with selected immune cell subsets. The **hypodermis** is the third layer of the skin and acts as an energy reserve and isolation for thermoregulation (J. Wang et al., 2025). It also immune cells and adiposis with production of a variety of inflammatory mediators (Weisberg et al., 2003).

1.4.2 Tissue-resident memory T cells of the skin

T cell polarization describes the combined functional state of a T cell, reflected by its cytokine profile, migratory potential, differentiation state, and effector functions (Masopust et al., 2026). Upon activation, most effector T cells undergo **activation-induced cell death**, but a small fraction survives and can commits to a **long-lived memory** T cell fate (L. Zhang & Romero, 2018). Memory T cells are difficult to distinguish from other antigen-experienced T cells, but

characteristic markers have been defined in both humans (CD11a^{hi}, often CD95⁺, CD58⁺, and CD49d⁺) and mice (CD44^{hi}, CD11a^{hi}, and typically CD127⁺) (Masopust et al., 2026). The two principle subsets of memory T cells are **central memory T** (TSM) cells and **effector memory T** (TDM) cells (Sallusto et al., 1999). TSM cells express CCR7 and patrol secondary lymphoid organs. Upon reactivation, they rapidly proliferate and give rise to effector T cells. TDM cells lack CCR7 and patrol peripheral tissues. Although they have less proliferative capacity than TSM cells, they exhibit a lower activation threshold and rapidly produce large amounts of effector molecules. Subsequently, **tissue-resident memory T cells** (TDRM) were identified following the recognition that subsets of CD4⁺ and CD8⁺ T cells persist long term in peripheral tissues such as the lung, skin, and gut without recirculating (Figure 5) (Szabo et al., 2019).

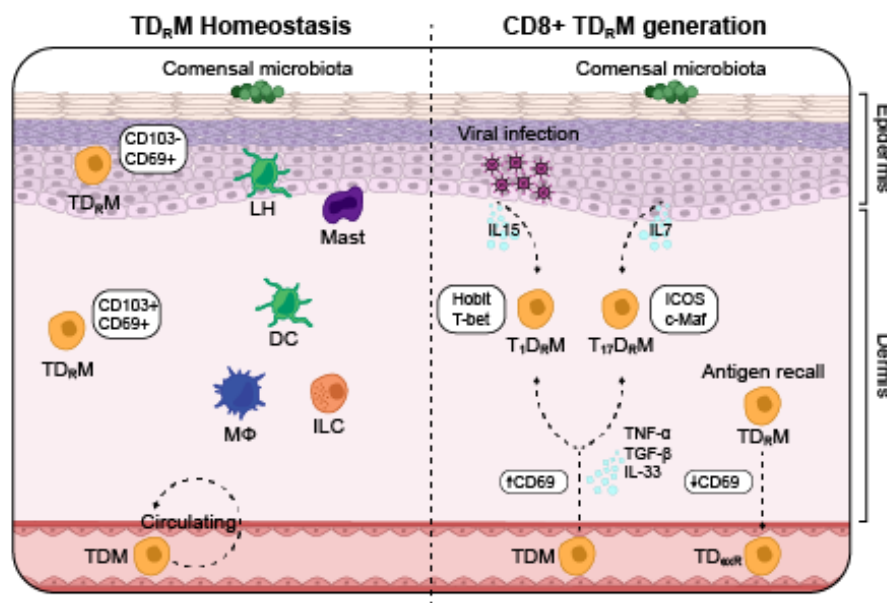


Figure 5 Principles of skin TDRM homeostasis and CD8+ TDRM generation.

The left panel shows a simplified representation of skin architecture and immune-cell populations, omitting additional anatomical structures such as hair follicles and glands as well as immune-cell subsets. The right panel summarizes principal pathways of T₁DRM and T₁₇DRM generation described in mice and highlights retrograde migration upon antigen recall. Abbreviations: DC, dendritic cell; ILC, innate lymphoid cell; LC, Langerhans cell; MΦ, macrophage; TDM, circulating effector memory T cell; TDRM-ex, TDRM-derived ex-resident memory T cell; TDRM, tissue-resident memory T cell; T₁DRM, type 1-polarized TDRM; T₁₇DRM, type 17-polarized TDRM.

The mechanisms underlying tissue residency and long-term maintenance are increasingly understood but remain incompletely resolved. Most insights have been derived from mouse studies and are only partly transferable to humans, as human studies are largely limited to blood samples or to pathological tissue obtained from transplant recipients and individuals with disease. Likewise, mouse studies are typically performed under highly controlled, pathogen-limited conditions, which may obscure important aspects of TDRM biology. In human skin, the dermis is enriched for CD103⁻ CD4⁺ TDRM, whereas the epidermis contains both CD4⁺ CD103⁺ and CD8⁺ CD103⁺ TDRM. By contrast, the mouse epidermis is dominated by CD103⁺

CD8⁺ T_{DRM}, while dermal CD4⁺ T_{DRM} display greater recirculatory capacity (N. Collins et al., 2016; Hochheiser et al., 2021; Watanabe et al., 2015).

In both mice and humans, a key mechanism of tissue retention is the inhibition of S1PR1 by CD69, which prevents T cells from exiting tissues in response to sphingosine-1-phosphate (SP1) gradients (Mackay, Braun, et al., 2015; Shioh et al., 2006). This requires downregulation of KLF2 by tissue cytokines such as TNF- α , TGF- β or IL-33 and maintenance enforced by IL-15 and IL-7 (Stanley Cheuk et al., 2014; Mackay et al., 2013; Skon et al., 2013). In mouse T_{DRM} development depends on downregulation of Eomes and T-bet and upregulation of Hobit and Blimp-1 (Mackay, Wynne-Jones, et al., 2015). Humans T_{DRM} do also express Hobit and Blimp-1 but at lower levels (Poon et al., 2023). The first polarized skin T_{DRM} subsets to be defined in humans were CD49a⁺ CD8⁺ T₁**DRM** and CD49a⁻ CD8⁺ T₁₇**DRM**, and subsequent work showed that cytotoxic CD49a⁺ CD8⁺ T_{DRM} depend on the RUNT family transcription factors RUNX2 and RUNX3 (S. Cheuk & Schlums, 2017; Zitti & Hoffer, 2023). In mouse, it was later shown that, following herpes simplex virus infection, CD8⁺ T₁DRM depend on Hobit, IL-15, and T-bet, whereas after *Staphylococcus epidermidis* challenge, CD8⁺ T₁₇DRM are characterized by IL-17A and ICOS expression and require c-Maf and IL-7 for their development (S. L. Park & Christo, 2023). The CD4⁺ T₁DRM and T₁₇DRM populations have also been described in mice, but in contrast to CD8⁺ T_{DRM}, their distinct developmental requirements remain incompletely defined, and evidence in humans is even more limited (Bartolomé-Casado, 2021; Y. Zou & Yuan, 2021). Long-lived tissue residency of T_{DRM} requires metabolic adaptation. Most notably, T_{DRM} depend on exogenous lipid uptake and fatty acid oxidation and reduced mitochondrial respiration to support their long-term survival and function. (Pan et al., 2017; Villa & Sanin, 2024). Adaptation to tissue residency was initially viewed as a terminal differentiation state. More recent work, however, indicates that T_{DRM} can undergo transdifferentiation during **retrograde migration** (T_{DRM}^{exR}) and home to other organs (Behr & Parga-Vidal, 2020; Fonseca et al., 2020; Klicznik & Morawski, 2019). Mouse studies have shown that up to 30% of circulating CD8⁺ T cells after primary infection are derived from former T_{DRM}, suggesting that retrograde migration substantially contributes to outside-in immunity and blood memory T cell function (Wijeyesinghe et al., 2021). Conversely, in humans, transdifferentiation has been described predominantly for CD4⁺ T_{DRM} under homeostatic conditions (Klicznik & Morawski, 2019).

1.4.3 Hematopoietic stem cell transplantation and graft versus host disease

The most important function of T_{DRM} is to provide a rapid and efficient local recall response upon recurrent pathogen encounter and tumor immune responses (Muruganandah et al., 2018; Okla et al., 2021). However, T_{DRM} can also contribute to pathology in inflammatory skin diseases such as psoriasis and atopic dermatitis, where they act as major producers of

cytokines that sustain inflammatory circuits and promote tissue damage (L. Chen & Shen, 2020). Autoreactive CD8⁺ T_H17 are key drivers of autoimmune tissue damage, as they mediate host-cell killing, produce high levels of IFN- γ , and can amplify inflammatory circuits through IL-15-dependent interactions with macrophages (Ryan et al., 2021).

The role of T_H17 in graft-versus-host disease (GVHD) following hematopoietic stem cell transplantation (HSCT) is complex and remains poorly defined. Myeloablative conditioning is a vital component of HSCT-based therapeutic strategies for both malignant (e.g., myeloid leukemia, myelodysplastic syndrome, acute lymphoblastic leukemia) and non-malignant (e.g., thalassemia, sickle cell disease, primary immunodeficiencies) disorders. (Kanate et al., 2020). It eradicates diseased hematopoietic cells but also depletes healthy immune cells, thereby providing profound immunosuppression, reducing GVHD rejection, and creating space within hematopoietic niches for donor HSC engraftment (Shimoni et al., 2024). Although high-dose chemotherapy with or without total body irradiation may reduce relapse relative to lower-intensity conditioning, these regimens also cause greater tissue damage and regimen-related toxicity, and the resulting sterile inflammation is an important determinant of HSC engraftment (Peci et al., 2022; Shimoni et al., 2024). HSCs can be collected directly from the bone marrow, from umbilical cord blood after birth, or from mobilized peripheral blood by apheresis (Kenyon & Babic, 2023; Z.-L. Xu & Huang, 2021). Compared with allogeneic HSCT, autologous and syngeneic HSCT carry a substantially lower risk of GVHD, but this is accompanied by reduced graft-versus-tumor activity and an increased risk of relapse (Aljaghtmi & Abdel-Aziz, 2025; Barisic & Childs, 2022). GVHD is a multi-system inflammatory disease with high mortality rate and is classified based on its clinical manifestations into acute (aGVHD) and chronic (cGVHD) forms (Tamaki et al., 2024). Whereas cGVHD is characterized by pleiotropic multi-organ manifestations and involvement of B cells (Zeiser & Blazar, 2017b), aGVHD develops in epithelial and T_H17 rich organs with most frequent onset detected in the skin followed by gastrointestinal tract and liver (Zeiser & Blazar, 2017a). Importantly, myeloablative conditioning does not achieve complete donor chimerism in peripheral tissues, and host T_H17 can persist in human skin for up to 10 years after transplantation (Strobl et al., 2020). The following section focuses on the pathophysiology and role of T_H17 in aGVHD, as this is the form investigated in the publication discussed in this thesis. Clinical onset of aGVHD is assessed by the presence of a maculopapular rash, hyperbilirubinemia, nausea, vomiting, and anorexia. The first trigger of aGVHD is the tissue damage induced by the myeloablative conditioning, accompanied by microbial dysbiosis (Jansen et al., 2022) and viral reactivation (Tong & Worswick, 2015). Excessive release of PAMPs and DAMPs activate innate immune cells which amplify inflammatory signals. In parallel, host APCs migrate to the SLO where they activate alloreactive donor T cells (Merad et al., 2004; Zeiser & Blazar, 2017a). In allogeneic HSCT, major HLA matching reduces the risk of GVHD, although it may also diminish graft-versus-

tumor activity (Barisic & Childs, 2022). Mismatches in MHC class I chain-related gene A (MICA) are associated with high risk of GVHD while the contribution of minor histocompatibility antigen mismatches is more complex (Carapito et al., 2016; Martin et al., 2017). Following activation, donor T cells clonally expand and disseminate through the circulation to epithelial target organs, where cytotoxic injury, cytokine amplification, and progressive barrier dysfunction promote microbial translocation and pathogen reactivation and thereby drive the high morbidity and mortality of severe aGVHD (Beilhack et al., 2005; Malard et al., 2023). Although the skin is often the first clinically involved organ, activated donor T cells subsequently spread to the gastrointestinal tract and liver, where local antigen presentation and inflammatory chemokine circuits sustain multi-organ disease (Zeiser & Blazar, 2017a). First-line treatment of aGVHD relies on systemic corticosteroids and other blocker of inflammatory signaling (Tamaki et al., 2024). During the effector phase of acute graft-versus-host disease, first-line treatment is systemic corticosteroids, whereas steroid-refractory disease often requires second-line immunosuppression, including ruxolitinib (Penack et al., 2024). However, whether donor-derived T cells that enter the skin after HSCT under conditions of tissue injury and inflammation acquire bona fide TDRM identity, and how failure or dysregulation of this process contributes to aGVHD, remained unresolved and is investigated in the second manuscript of this thesis (Chapter 2.2)

1.5 Computational modelling of immune cell state transitions

Gregor Mendel demonstrated that biological inheritance follows quantifiable principles and that phenotypic outcomes can be predicted statistically (Abbott & Fairbanks, 2016). The molecular substrate of this inheritance is deoxyribonucleic acid (DNA), which is present in every known cellular organism and encodes the regulatory potential from which cellular identity and function emerge (Franklin & Gosling, 2003; Watson & Crick, 1953). The **Human Genome Project** (1990-2003), driven publicly by the International Human Genome Sequencing Consortium under Francis Collins, prevailed over the private sequencing effort of J. Craig Venter's Celera Genomics and established the human genome as an openly accessible reference for biomedical research (Lander et al., 2001). At the same time, this competition demonstrated that commercial incentives can be powerful drivers of technological innovation, enabling scientific discoveries to be translated into scalable technologies (F. S. Collins et al., 2003). In the following decades, genome sequencing expanded across species and revealed the deep evolutionary relationships between organisms. Beyond their philosophical implications, these discoveries and the functional annotation of the genome enabled disease mechanisms to be studied at molecular scale (Dash & Mestre, 2020; King, 2014; Rommens et al., 1989). Early genome-wide approaches relied on microarrays and later bulk high-throughput sequencing of whole tissues or sorted populations, generating large datasets but averaging signals across

heterogeneous cell mixtures. The development of **single-cell RNA sequencing** enabled gene expression to be measured at the level of individual cells and led to initiatives such as the **Human Cell Atlas** (Kalisky & Quake, 2011; Regev et al., 2017; Tang et al., 2009). This transformed tissues from experimentally averaged entities into single-cell resolved biological networks. Current methods no longer capture transcriptomes alone, but increasingly integrate multimodal single-cell measurements, including chromatin accessibility, DNA methylation, histone modifications, protein abundance, spatial localization, immune receptor repertoires, lineage tracing, and CRISPR–Cas perturbation screens (Baysoy et al., 2023). The resulting high-dimensional datasets require substantial computational resources and specialized algorithms for interpretation. This adds yet another layer to the increasingly multidisciplinary nature of biomedical research and underscores the need for specialized bioinformatics expertise (Lähnemann et al., 2020; Way et al., 2021). Together, these developments illustrate how **bioinformatics** has moved beyond a downstream analytical role and is increasingly integrated into hypothesis generation, experimental design, and mechanistic interpretation. The following chapter outlines principles of single-cell RNA-seq transcriptomics computational modeling used to capture cell state transitions and functional polarization in health and disease, with relevance to the publications arising from this work.

1.5.1 Graph-based modeling of cellular states

Single-cell technologies capture transcriptomic profiles from thousands to millions of individual cells, typically represented as a cell-by-gene count matrix. Early single-cell RNA-seq studies primarily relied on principal component analysis and hierarchical clustering strategies to identify transcriptionally similar cells (Buganim et al., 2012; G. Guo et al., 2010). As datasets increased in size and complexity, **nearest-neighbor graphs** emerged as a scalable representation, in which cells are first embedded into reduced feature spaces and then connected according to local transcriptomic similarity (Cover & Hart, 1967; Eppstein et al., 1997; Levine et al., 2015; Satija et al., 2015; C. Xu & Su, 2015). In these cell–cell neighborhood graphs, nodes correspond to individual cells and edges are derived from pairwise distances or similarities, such as Euclidean distance in principal component space, Pearson correlation, or shared-neighborhood overlap measured by Jaccard similarity. Such graphs provide a computational approximation of **cellular state space** and form the basis for clustering, trajectory inference, and visualization. However, graph-based representations depend on multiple statistical and technical assumptions (Lähnemann et al., 2020). (i) Most high-throughput single-cell RNA-seq protocols capture only a shallow fraction of each cell's transcriptome. This results in sparse count matrices with many zero values (often >90% of the counts), which may reflect true absence of expression or technical non-detection caused by limited and stochastic transcript capture. Distinguishing biological zeros from technical non-

detection therefore remains a central challenge in single-cell data analysis (R. Jiang et al., 2022). (ii) Single-cell datasets are intrinsically high dimensional. Although modern experiments may profile thousands or even millions of cells, each cell is measured across many thousands of genes. Direct interpretation in this original gene space is unstable because distances become less informative, noise accumulates across dimensions, and the number of features can exceed the number of independent observations. This phenomenon is commonly referred to as the curse of dimensionality and motivates the use of feature selection prior to dimensionality reduction for graph construction (Germain et al., 2020; Hall et al., 2005; Tsuyuzaki et al., 2020). (iii) Variation in gene expression can arise from both biological processes and technical sources. Technical variation may reflect differences in library preparation protocols, sequencing depth, sample storage or tissue dissociation protocols. The latter is particularly relevant for immune cells, because enzymatic digestion, temperature, and processing time can induce stress or activation programs even before cDNA capture (Sikkema et al., 2023). At the same time, not all unwanted variation is technical. Depending on the biological question, patient-specific transcriptional programs, cell cycle effects or mitochondrial content may either represent meaningful biology or confounding variation. Thus, graph-based modeling requires careful distinction between biological signals, technical noise, and context-dependent covariates. To address these challenges, single-cell workflows often include data harmonization. The central objective of these methods is to reduce unwanted technical variation while preserving biological structure (Luecken et al., 2022). In this context, **deep generative models** provide an attractive framework for learning representations of single-cell data. Single-cell variational inference (scVI) models unique molecular identifier (UMI) count data probabilistically using a **variational autoencoder** and learns a low-dimensional latent representation that accounts for sequencing depth, overdispersion, and batch-associated variation (Lopez et al., 2018; Gayoso et al., 2022). Instead of constructing neighborhood graphs directly from normalized expression values or principal components, graphs can be computed from the scVI latent space, which aims to preserve biological structure while reducing unwanted technical variation. This strategy was central to the publications included in this thesis, where scVI latent representations were used for k-nearest-neighbor graph construction. Graph representations of single-cell transcriptomes become particularly powerful when cellular identity is dynamic rather than discrete. In contrast to whole-tissue transcriptomics or bulk RNA sequencing of FACS-sorted populations, graph-based single-cell analysis positions individual cells within continuous state spaces without requiring prior marker-based definitions, thereby enabling unbiased discovery of previously unrecognized cell states in health and disease (Domínguez Conde et al., 2022; Sikkema et al., 2023; Tabula Muris Consortium, 2020; Tabula Muris Consortium et al., 2018; Tabula Sapiens Consortium* et al., 2022).

1.5.2 Pseudotime trajectories and the challenge of directionality

For downstream analysis, the cellular graph is often partitioned by stratifying cells into discrete subsets. While this strategy is useful for identifying major cell types, it can impose artificial boundaries on biological processes that unfold gradually, such as differentiation, proliferation, tissue adaptation, activation, or functional polarization. **Trajectory inference** methods therefore complement clustering by using the topology of single-cell graphs to model relationships between cellular states, including linear progressions, bifurcations, tree-like hierarchies, cyclic processes, and disconnected trajectories (Saelens et al., 2019; Trapnell et al., 2014). **Pseudotime** provides a relative ordering of cells along one or more reconstructed trajectories and, when anchored to a biologically defined root state, can orient the cellular graph along an inferred axis of biological progression. It does not represent chronological time but estimates progression through a biological process based on transcriptional similarity and graph connectivity, allowing gradual changes in gene expression, regulatory activity, or cellular phenotype to be analyzed along directed state transitions. Pseudotime methods differ primarily in how they define progression through cellular state space. Principal-trajectory approaches reduce the high-dimensional manifold to one or several curves or tree-like structures and order cells by their position along these inferred axes (Street et al., 2018; Trapnell et al., 2014). Diffusion-based methods instead use random-walk distances on the cellular graph, such that pseudotime reflects gradual connectivity through regions of high cell density rather than direct distance in expression space (Haghverdi et al., 2016). Graph abstraction model transitions between connected cell-state communities, preserving global topology while reducing sensitivity to noise (Wolf et al., 2019). Other approaches infer developmental progression from transcriptional diversity, based on the observation that the number of detectably expressed genes tends to decline during differentiation (Gulati et al., 2020). RNA velocity builds on the observation that the relative abundance of unspliced and spliced transcripts in single-cell RNA sequencing data contains information about transcriptional dynamics, which can be used to estimate the future expression state of individual cells and infer the direction of cellular state transitions without requiring a manually defined root state (Bergen et al., 2020; La Manno et al., 2018). In parallel, probabilistic trajectory models emerged that represent cell state transitions as a Markov process over the cellular graph, estimating transition probabilities between neighboring cells (Setty et al., 2019). However, no single trajectory model is suitable for all forms of cellular progression, and the choice of method must be matched to the biological system and the assumptions that can be justified. Recent approaches have therefore shifted toward integrating multiple sources of transition information rather than relying on a single model of cellular progression. CellRank implements this idea through transition kernels that encode distinct assumptions about cellular dynamics, including transcriptomic similarity, RNA-velocity, pseudotime, experimental time points, or lineage-tracing information (Lange et al.,

2022; Weiler et al., 2024). Together, these models provide the analytical basis for examining how inflammatory perturbation reshapes cellular state transitions in the splenic erythroid niche during stress erythropoiesis and in the human skin T cell compartment following HSCT and GVHD.

1.6 Aim of the thesis

Over the past decade, tissue-specific signaling, functional states, and embryonic origins of tissue-resident macrophages have been defined in increasing detail. However, many open questions remain regarding the conditions that promote the generation of tissue-resident macrophages in adult individuals, particularly during inflammation. In parallel, erythrocytes are increasingly viewed as active participants in immune responses, and dedicated stress erythroid progenitors have been defined in the mouse spleen. While inflammatory signals suppress bone marrow erythropoiesis, it has become clear that such signals must also be integrated into stress erythroid progenitor expansion. However, how these signals regulate erythropoiesis, and when they may become pathogenic and contribute to anemia of inflammation, remains incompletely understood. In the first study (Chapter 2.1), we present a fully integrated, time-resolved single-cell model of the erythroid niche during stress to address the following aims:

Aim 1 – Identify the inflammatory signals that drive monocyte-derived RPM development.

Aim 2 – Define how myeloid niche cells communicate with stress erythroid progenitors.

Aim 3 – Determine how erythroid progenitors integrate inflammatory signals.

Following myeloablative conditioning, host TD_RM can persist for decades in human skin, indicating that cutaneous immune memory is not fully erased by hematopoietic replacement. Mouse studies further suggest that distinct TD_RM subsets arise through different differentiation and maintenance pathways. In the second study presented in this thesis, we therefore investigated mechanisms of human TD_RM biology by modeling TD_RM niche reconstitution after HSCT and during aGVHD. Using longitudinal skin and blood sampling, we aimed to define how the post-transplant skin niche shapes TD_RM heterogeneity, persistence, and inflammatory remodeling.

Aim 1 – Resolve host–donor chimerism at single-cell resolution.

Aim 2 – Dissect reconstitution dynamics between blood and skin after HSCT.

Aim 3 – Identify markers of TD_RM retrograde migration during aGVHD.

2 Results

2.1 Myeloid IFN-I sensing boosts stress erythropoiesis

Title:

Myeloid type I IFN sensing boosts splenic stress erythropoiesis

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ABSTRACT

Inflammation is known to disrupt steady-state bone marrow erythropoiesis while activating stress erythropoiesis in extramedullary organs like the spleen, to preserve red blood cell production. How inflammatory cues are temporally coordinated within the splenic erythropoietic niche remains unclear. Here we combine time-resolved single-cell RNA sequencing with trajectory modeling and gene regulatory network inference to define the role of myeloid type I interferon (IFN) sensing during CpG-induced stress erythropoiesis. We found that loss of myeloid IFN sensing specifically hampered splenic erythroblast expansion and stress-erythropoiesis associated splenomegaly, while seemingly not affecting the bone marrow erythroid compartment. Mechanistically, IFNAR-deficient monocytes failed to fully acquire a red pulp macrophage transcriptional phenotype after inflammatory challenge, with reduced SPI-C activity, impaired iron-handling programs and diminished GDF15 expression. Erythroid trajectory modeling further revealed a stress-induced IFN-response signature that was attenuated at later time points in *Ifnar1^{fl/fl} LysM^{cre/+}* mice, coinciding with a transcriptional shift toward EPOR–JAK–STAT associated terminal differentiation. These findings identify myeloid type I IFN sensing as a niche specific requirement for temporal amplification of splenic stress erythropoiesis that links monocyte-derived niche adaptation to sustained erythroid progenitor activation.

INTRODUCTION

Inflammation suppresses steady-state bone marrow erythropoiesis while promoting emergency myelopoiesis¹⁻⁴. To preserve red blood cell homeostasis, mice activate a compensatory stress erythropoiesis (SE) program that is largely extramedullary and centered in the spleen. Within the spleen, red pulp macrophages (RPMs) perform two key functions: they form erythroblastic islands with differentiating erythroid cells to support erythroblast maturation, and they recycle iron through phagocytosis of senescent erythrocytes^{5,6}. Failure of SE during inflammation can contribute to anemia of inflammation, a clinically significant complication of chronic infection, cancer, and autoimmune disease⁷.

Acute-phase pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1) and interferon- γ (IFN- γ), as well as tissue-damage-associated signals such as IL-33, suppress steady-state bone marrow (BM) erythropoiesis¹⁻⁴. Early induction of SE is associated with increased erythrophagocytosis by splenic red pulp macrophages (RPMs)⁸. While IFN- γ promotes macrophage phagocytic activity, various pathogen- and damage-associated signals can disrupt the protective interaction between red blood cell CD47 and SIRP α on RPMs⁹⁻¹². While RPMs are predominantly embryonically derived in unchallenged mice, increased erythrophagocytosis during stress elevates heme levels, promoting the differentiation of recruited monocytes into RPM-like cells through heme-dependent activation of SPI-C^{13,14}. In parallel, bone morphogenetic protein 4 (BMP4)-dependent stress erythroid progenitors (SEPs) expand, giving rise to stress BFU-E and later CFU-E populations that engage with monocytes and RPMs within the erythroid niche^{15,16}.

While inflammatory signals are sufficient to initiate stress erythropoiesis pathways, chronic inflammation suppresses erythroid proliferation and disrupts systemic iron homeostasis¹⁷. However, how inflammatory signals are temporally coordinated to support the transition from induction to sustained stress erythropoiesis remains unclear. In this study, we generated time-resolved single-cell data from the splenic erythropoietic niche at steady-state and in a model of CpG-ODN induced stress erythropoiesis to investigate how inflammatory signaling is integrated during stress erythropoiesis. We find that early myeloid type I IFN sensing is required for the development of monocyte-derived RPMs and that its loss markedly impairs red blood cell production. Erythroid progenitors initially display a type I IFN response, but this response is not maintained in myeloid IFNAR-deficient mice and is associated with induction of hypoxia programs and *Erfe* expression.

RESULTS

Stress erythropoiesis activates an IFN-responsive gene program

To dissect steady-state versus stress erythropoiesis programs, we induced extramedullary erythropoiesis with CpG-ODN^{18,19} and captured transcriptional dynamics within the stress erythropoietic niche by single-cell RNA sequencing (scRNA-seq) in control spleens and on day six after CpG challenge, at the peak of erythroblast expansion¹⁸. Prior scRNA-seq, cells were sorted to enrich mononuclear phagocytes, erythroid cells and precursors (Figure 1A). We recovered 24,954 high-quality single-cell transcriptomes, which upon integration revealed 17 distinct cell clusters (Figure 1B).

We identified classical dendritic cells type 1 (cDC1; *Flt3*, *Xcr1*, *Cd8a*) and type 2 (cDC2; *Sirpa*, *Flt3*, *Cd4*), migratory cDCs (cDC mig.; *Ccr7*), and monocyte derived cDC (moDC; *Cd209a*), classical (cMo; *Ly6c2*), intermediate (intMo; *Ly6c2*, *Cx3cr1*), and non-classical (ncMo; *Cx3cr1*) monocytes, as well as red pulp macrophages (RPMs; *Adgre1*, *Spic*, *Cd163*)^{13,14} and basophils (*Cebpa*, *Itga2*). Amongst progenitors and stem cells (*Cd34*, *Kit*) we distinguished granulocyte-monocyte progenitors (GMPs; *Spi1*), neutrophil- (NeuP; *Ela*), basophil- (BasoP; *Cebpa*, *Mcp1*, *Clec12a*), mast cell- (MastP; *Mcp1*, *Cma1*) as well as megakaryocyte-erythroid progenitors (MEPs) (Figure 1B and S1A). The latter constitutes the largest progenitor cluster in our data set and is characterized by low level expression of megakaryocyte progenitor (MegP; *Pf4*, *Itga2b*) and erythroid lineage (*Gata1*, *Klf1*) marker genes but also shares some overlap with granulocyte lineages (Figure S1A and S1B). In addition, we identified a cluster of committed erythroid progenitors resembling a CFU-E state, marked by a downregulation of progenitor markers (*Kit*, *Meis1*) and a switch from *Gata2* to the master erythroid transcription factors *Gata1* and *Klf1* (Figure S1A and S1B). The second erythroid cluster is enriched for erythroblasts (EB) with *Sox6* driven induction of hemoglobin synthesis (*Alas2*) and increased iron uptake (*Tfrc*). We exclusively detect hemoglobin genes of definitive (*Hba-a1*, *Hba-a2*, *Hbb-bt*, *Hbb-bs*) but not primitive (*Hba-x*, *Hbb-y*, *Hbb-bh1*) erythropoiesis. At the late stages of the erythroid trajectory, hemoglobin transcripts accounted for ~30% of total expression (Figure S1B), suggesting a representation of cells up to the orthochromatic erythroblast stage²⁰.

Differential abundance testing of populations confirmed a pronounced expansion of erythroblasts upon CpG-induced stress erythropoiesis (Figure 1C) and CpG-ODN stimulation reorganized the splenic myeloid progenitor pool, increasing GMPs, NeuPs, and BasoPs while reducing MegPs and MastPs. Monocytes, RPM and DC showed similar average abundances between conditions (Figure 1C), yet differential abundance testing indicated distinct neighborhood enrichment with numerous differentially expressed genes (DEGs) (Figure 1D). Even six days post CpG challenge, IFN response genes like *Ly-6a*, *Ifit*, *Gbp* or MHC transcripts

were still upregulated in myeloid cell populations, along with an enhanced expression of oxidative phosphorylation genes (Figure 1E). RPMs showed a downregulation of lineage-specific genes (*Spic*, *Mertk*), disrupted iron homeostasis (*Slc40a1*, *Hmox1*, *Gdf15*), and an upregulation of factors linked to phagocytosis (e.g. *Msr1*, *Marco*, *Fcgr2b*) and antigen presentation (*H2-Eb1*, *H2-Ab1*) (Figure 1F). Cell density distribution analysis indicated that RPMs on day 6 do not fully reconstitute the RPM niche (Figure S1C) and together these data showed that in CpG-stimulated spleens, RPMs are monocyte-derived and not yet fully tissue-adapted, as previously described^{8,21}.

Amongst precursors and stem cells, most DEGs were found in CFU-Es, followed by MEP and EBs, the other two erythroid cell populations (Figure 1D). For advanced modeling of transcriptional dynamics across continuous differentiation trajectories²², erythroid development was reconstructed using diffusion pseudotime (DPT) (Figure 1G). In total, 8,950 genes exhibited dynamic expression patterns during erythroid maturation, of which the majority (8,060 genes) were not differentially expressed between steady-state and day 6 of stress erythropoiesis and were thus defined as canonical (Figure 1H). Smoothed expression profiles of canonical genes were grouped according to their expression maxima, identifying modules peaking at the MEP (1,582 genes), CFU-E (5,766 genes), or erythroblast (712 genes) stages (Figure 1I and 1J). The model faithfully recapitulated the sequential regulation of key transcription factors along the erythroid lineage. *Meis1* and *Gata2* were progressively downregulated, whereas *Gata1*, *Klf1*, and *Tal1* were induced during erythrocyte commitment. Early in this transition, epigenetic modifiers (*Dnmt3b*, *Tet2*, *Tet3*) were upregulated, coinciding with extensive remodeling of metabolic programs. Cells shifted from oxygen-restricted glycolysis (*Hif1a*, *Ldha*, *Pkm*, *Pdk1*) toward oxidative phosphorylation (*Atp5a1*, *Atp5b*, *Sod2*), mitochondrial biogenesis (*Tfam*, *Ssbp1*, *Polg*), and β -oxidation (*Cpt1a*, *Acadl*), accompanied by increased activity of the pentose phosphate (*G6pd*, *Pgd*) and mTOR (*Mtor*, *Rptor*, *Rheb*) pathways. During the CFU-E stage, *Kit* expression declined as *Epor* levels rose. Subsequently, receptors involved in erythroblastic island formation (*Icam4*), iron trafficking (*Tfrc*, *Ncoa4*)²³, and hemoglobin synthesis (*Alas2*) were induced, alongside genes mediating mitochondrial clearance (*Bnip3l*). Together, these findings indicate that the core differentiation programs are shared between splenic steady-state and stress erythropoiesis.

Interestingly, roughly 10% of genes (890) showed a differential regulation between conditions, with most of them, 568, being downregulated and 184 genes, being upregulated during CpG-induced erythroid differentiation (Figure 1H and 1K). Amongst downregulated genes, we found HES1, which is typically repressed by GATA1 to promote early erythroid progenitor to erythroblast differentiation²⁴ and genes involved in cholesterol metabolism (*Ldlr*, *Hmgcr*)²⁵. Notably, a set of IFN (IFN) response signature genes peaked at the CFU-E stage (*Stat1*, *Stat2*,

Gbp3/7) in the stress condition and early stress erythroblasts increased *Gbp8* which was completely absent in the steady state. In addition, IFN-inducible *Apol11b*, implicated in lipid metabolism²⁶, was strongly induced towards the end of erythroblast differentiation in the CpG group. While *Eif2ak2*, another key ISG, seemed to be suppressed towards the end of the stress erythroid trajectory, these data together showed that even six days post CpG-challenge, erythroid populations still showed traces of IFN exposure, which has likely happened earlier.

Myeloid Type I IFN sensing boosts extramedullary erythroblast expansion

TLR9 stimulation with CpG-ODN typically triggers type I IFN production by tissue-resident macrophages such as RPMs, while simultaneously promoting heme-driven maturation of newly recruited monocytes into RPM-like cells by boosting erythrophagocytosis. While IFN-signaling is classically viewed as inhibitory for bone-marrow erythropoiesis^{27,28}, the long-lasting IFN-responsive program we observed in splenic erythroid cells led us to hypothesize that local myeloid cell IFN responses may serve a distinct, underappreciated function during inflammation-induced stress erythropoiesis. To test this hypothesis, we therefore made use of mice lacking the type I IFN receptor specifically in the myeloid cell compartment (*Ifnar1^{fl/fl} LysM^{cre/+}*) and will therefore not amplify their IFN response upon CpG challenge (Figure 2A).

When we first analyzed the spleens of these mice post CpG-injection, our first observation was an impressive reduction of splenomegaly in animals compared to *Ifnar1^{fl/fl} LysM^{+/+}* littermate controls. The difference was seen as early as D3 post injection and got more pronounced by D6 (Figure 2B). Importantly, the reduction in spleen size was not attributable to decreased leukocyte abundance, as total leukocyte numbers and major subsets were maintained or even increased in *Ifnar1^{fl/fl} LysM^{cre/+}* spleens (Figure 2C-I, S2A). In contrast, *Ifnar1^{fl/fl} LysM^{cre/+}* mice exhibited a marked impairment in erythroblast expansion by day 6 (Figure 2J, 2K and S2B) despite elevated RPM counts (Figure 2I). Importantly, bone marrow erythroblast numbers did not differ between genotypes, and leukocyte populations remained comparable following CpG challenge (Figure S2C-G), suggesting that observed differences in the spleen did not result from differences in bone marrow erythropoiesis suppressed by CpG. Further, the distribution of terminal differentiation stages, herein classified as stage I (proerythroblast/very early erythroblasts enriched fraction) to stage IV (orthochromatic erythroblast enriched fraction) followed an expected 1:2:4:8 ratio²⁹ (Figure S2H). Together, these data showed that myeloid type I IFN sensing promoted Erythroblast expansion under stress conditions without directly affecting terminal differentiation of erythroblasts. Along with this, transcriptomes from sorted erythroblasts remained comparable between genotypes to baseline across all time points (Figure S2I-K), suggesting that myeloid IFNAR deficiency did not impact terminal erythrocyte differentiation directly, but may rather affect erythroblast survival, turnover or earlier progenitor stages.

Unaffected erythroid MyD88 responses and metabolic reprogramming in myeloid IFNAR deficiency

To gain deeper mechanistic insights, we next performed scRNA-sequencing of sorted cells from spleens from *Ifnar1^{fl/fl}* *LysM^{cre/+}* and control mice at early time points post CpG challenge (Figure 3A, Figure S3A and S3B). Diffusion pseudotime analysis (Figure 3B and 3C), revealed transcriptional responses to CpG-ODN treatment in both groups on day 1 and day 3 (Figure 3D). As observed in our earlier comparison between control samples and day 6 after CpG-ODN challenge, most genes (6,118) exhibited stable expression across time points and genotypes (Figure S3C). To define shared stress-response programs, we first excluded genotype-dependent genes and identified 1,200 upregulated (Figure 3E) and 400 downregulated transcripts (Figure 3F) on day 1 or day 3 compared to controls.

Splenic stress BFU-E were defined by the surface marker Lin⁻ c-Kit⁺ CD24a^{low} CD71^{low} CD150⁺ and further specified by CD9 and Sca1 expression³⁰. We found that the corresponding genes *Kit* (c-Kit), *Cd24a* (CD24a), *Tfrc* (CD71), and *Slamf1* (CD150) were canonically expressed, with transcript levels reflecting the expected surface marker distribution (Figure 3G). Expression of *Cd9* (CD9) was significantly increased by day 3, indicating expansion of stress BFU-E. Moreover, *Cd9* expression was limited to the progenitor stage, matching the reported loss of CD9 during transition to the CFU-E stage³⁰. In an irradiation model³⁰, *Ly6a* (Sca-1) expression was associated with multipotent progenitors rather than stress BFU-Es. In contrast, we observed high *Ly6a* expression at the progenitor and CFU-E stages, with reduced expression on day 3 in IFNAR-deficient mice (Figure 3G). In addition, we observed expression of survival genes important for splenic recovery following PHZ treatment (*Serpina3g*)³¹, and splenic repressor of erythroid terminal differentiation (*Lyf1*)³² at the progenitor stage (Figure 3G).

Myd88 was induced at the CFU-E stage (Figure 3H) and importantly, previous studies have shown that both human and mouse erythrocytes express TLR9 at the cell surface and intracellularly^{10,12}. Although *Tlr9* transcripts were not detected, core myddosome components (*Irk1/2/4*) and *Unc93b1* were expressed (Figure 3H). Alternatively, MyD88 is activated by IL-1 family cytokines but among IL-1 receptors only *Il1rl1/Il1rap* (ST2) were present (Figure S3D). Further we identified shared upregulation of a limited set of IFN-response and MHC class I genes (Figure S3E and S3F).

A hallmark of stress erythropoiesis is remodeling of metabolic programs to support rapid proliferation of erythroid progenitors³³. We observed upregulation (Figure 3I) of *Got1*, and genes involved in selenium metabolism (*Scly*)³⁴, the pentose phosphate pathway (*G6pdx*), glycolysis (*Pfkfb*), and lipid metabolism (*Acot9*, *Ldlrap1*, *Lrp8*) alongside downregulated (Figure

3J) of anabolic repressors (*Mxd4*, *Deptor*, *Txnip*, *Pdcd4*) consistent with coordinated activation of redox-balancing and anabolic metabolic programs. Genes related to oxidative phosphorylation were canonically induced at the CFU-E stage. However, we observed downregulation of the oxidative phosphorylation enhancer gene cytochrome c oxidase subunit 6B2 (*Cox6b2*)³⁵ and the mitophagy regulator Parkin (*Prkn*)³⁶ on day 1 and day 3 (Figure 3K).

Together, these data demonstrate that early erythroid stress responses, including MyD88-associated signaling and metabolic reprogramming, are largely preserved in the absence of myeloid IFNAR, indicating that initial CpG- induced activation of erythroid progenitors occurs independently of myeloid type I IFN sensing.

Sustained IFN and AP-1 transcriptional programs in erythroid progenitors require myeloid IFNAR signaling

We next compared the genotype groups and found that stress response on day 1 was comparable but differed by day 3 (Figure 4A). Inflammatory response pathways and IFN signaling pathways were activated in both groups (Figure 4B), but while inflammatory pathways did not differ between genotypes on day 1, they were significantly downregulated on day 3 in IFNAR-deficient mice (Figure S4A). This indicated that immediate, direct responses of the erythroid niche to CpG were similar between genotypes consistent with the observation of shared MyD88 activation.

We next asked which transcription factors were differently regulated during stress erythropoiesis and how myeloid IFNAR deficiency may affect erythroid transcription factor dynamics. Comparing transcription factor activity on day 1 and day 3 post CpG challenge to control samples revealed an enhanced activity of the transcription factors *Stat1*, *Stat2*, *Irf1*, *Irf7*, *Stat3*, *Atf3*, *Jun*, *Junb*, *Jund*, *Fos*, *Fosb* under stress, compared to homeostasis (Figure S4B). Hierarchical clustering identified two key transcriptional modules that differed between genotypes. The first module comprising *Stat1*, *Stat2*, *Stat3*, *Irf1*, and *Irf7*, was activated in MEPs and CFU-Es on day 1 and day 3 but cells failed to sustain this activation by day 3 in the absence of myeloid IFNAR (Figure 4C). The second module, consisting of *Atf3*, *Jun*, *Junb*, *Jund*, *Fos*, *Fosb* required myeloid IFNAR for induction in MEPs on day 3 post challenge (Figure 4C). Validation at the gene expression level confirmed significant activation and reduced expression on day 3 in IFNAR-deficient mice across all transcription factors examined (Figure 4D and 4E).

Having identified genotype-dependent differences in transcription factor activity, we next examined the downstream gene programs altered between genotypes on day 3. Of 513 perturbed genes, 311 were downregulated and 159 upregulated in myeloid IFNAR-deficient

mice compared to littermate controls (Figure 4A). To determine whether differentially expressed transcripts were linked to the altered transcription factor activity, we integrated expression profiles with regulatory network analysis (Figure 4F and 4G). The analysis revealed that transcripts downregulated in myeloid IFNAR-deficient mice on day 3 were strongly enriched for predicted targets of perturbed transcription factors, and their expression dynamics closely mirrored the two transcription factor modules (Figure 4F).

Erythroid progenitors exhibited a clear IFN response on day 1 that was not maintained in IFNAR-deficient mice by day 3. This was marked by activation of *Stat1*, *Stat2*, *Irf1*, and *Irf7* and enrichment of downstream IFN-stimulated gene programs (Figure S4C) and associated metabolic remodeling (Figure S4D). In addition to failed induction of AP-1 complex genes in myeloid IFNAR-deficient mice on day 3, we observed downregulation of NF- κ B/MAPK negative regulators (*Nfkbia*, *Nfkbid*, *Tnfaip3*, *Dusp1*) as well as immediate-early response genes (*Ier2*, *Ier5*, *Egr1*) at the early progenitor state (Figure S4E).

Interestingly, along with reduced IFN signaling at the CFU-E stage in myeloid IFNAR-deficient mice on day 3, we observed increased expression of EPO-inducible genes, including insulin receptor substrate 2 (*Irs2*)³⁷ and podocalyxin-like protein 1 (*Podxl*)³⁸, which are associated with EPO-driven proliferative and maturation-related programs. In addition, carboxypeptidase D (*Cpd*) and G protein-coupled receptor 150 (*Gpr150*) exhibited a similar expression pattern, although their roles in erythropoiesis remain poorly defined (Figure 4H and 4I). At the erythroblast stage, cytokine-inducible SH2-containing protein (*Cish*) and erythroferrone (*Erfe*)³⁹ were strongly upregulated in *Ifnar1^{fl/fl}* *LysM^{cre/+}* compared to control cells (Figure 4I).

Thus, while direct CpG responses seem present in erythroid cells, sustained IFN signaling and expression of AP-1 complex genes was hampered in the absence of myeloid IFNAR signaling, which went along with increased expression of genes linked to EPO-responsive erythroid activation and iron mobilization in erythroblasts.

Type I IFN sensing supports tissue adaptation of monocyte derived RPMs

We next examined myeloid cells as the likely source and regulators of these signals. We first computed the cluster-based number of response genes to baseline (Figure 5A) and between genotypes (Figure 5B). The analysis corroborated the erythroid trajectory results, with most response genes detected at the progenitor stage and genotype-specific differences emerging only by day 3. Myeloid cells efficiently responded to CpG, with most substantial, quickly induced transcriptional changes compared to baseline in cMo and intMo on day 1 (Figure 5A). Most pronounced differences between *Ifnar1^{fl/fl}* *LysM^{cre/+}* and littermate control animals were interestingly found in intMo on day 1, followed by cMo on day 3 post challenge. In contrast,

while CpG also induced substantial transcriptional changes in RPMs and different types of DCs, these populations did not show any differences between genotypes (Figure 5B).

Gene regulatory network (GRN) analysis of intMo revealed a strongly reduced activity of certain IFN-responsive (*Irf7*, *Stat2*, *Maf*) and metabolism-associated (*Spic*, *Nr1h3*) transcription factors, but an elevated signal for Stat1 activity in IFNAR deficient cells on day 1 (Figure 5C). Notably, the key transcription factor of red pulp macrophage development *Spic*^{13,14}, exhibited a high activity as early as day 1 post challenge, with a significantly reduced activation in *Ifnar1*^{fl/fl} *LysM*^{cre/+} cells compared to controls (Figure 5D) suggesting either a reduced enrichment of monocyte-derived preRPMs or a reduced differentiation of monocytes towards preRPM phenotypes within the intMo population at this time point²¹. Consistent with *Spic* induction in intMo on day 1, iron metabolism-associated pathways were upregulated in controls, but not in IFNAR-deficient cells (Figure 5E). Accordingly, we observed significant activation of iron metabolism and macrophage-associated genes in intMo (Figure 5F). While cMo also exhibited an RPM-like gene signature including *Spic* expression, it was less pronounced than in intMo, and SPI-C activity (Figure S5A) was not significantly increased across time points and genotypes.

The pathway and GRN analysis showed that CpG treatment induced pathways related to type I IFN production and response in control on day 1 and day 3 compared to IFNAR-deficient intMo (Figure 5C and 5E). Unexpectedly, type II IFN-responsive pathways were enriched in IFNAR-deficient intMo on day 1, but this effect changed by day 3. The differential expression of type I (*Irf7*, *Stat2*, *Ifit1*, *Ifit2*, *Ddx60*) and type II (*Ciita*, *Gbp2*, *Gbp2b*, *Gbp3*, *Gbp5*) IFN-response genes was consistent in both intMo (Figure 5F) and cMo (Figure 5G, Figure S5B). By day 3, reduced IFN responses were accompanied by a hindered induction of inflammatory response pathways in both IFNAR deficient monocyte subsets, including NF-κB, AP-1, and inflammasome signaling (Figure 3F and 3G). This was particularly pronounced in cMo with strongly reduced activity of respective transcription factors in myeloid IFNAR-deficient mice (Figure S5C). However, cMo and intMo differed in the cytokines they induced in response to CpG. The inflammatory cytokines *Tnf* and *Il1b* were selectively induced in cMos, whereas intMos upregulated the expression of immunomodulatory *Il18* and *Gdf15*⁴⁰ and importantly, in both cases the induction failed in *Ifnar1*^{fl/fl} *LysM*^{cre/+} cells, suggesting relevant crosstalk between type I IFN- and inflammatory signaling.

To gain insights into how deficient IFN sensing alters the erythroblastic niche interaction we performed cell-cell communication analysis focused on C-C motif chemokine ligands, C-X-C motif chemokine ligands, TNF and interleukins (Figure 5H). Notably, Tnf signaling via Tfrsf1a and Tfrsf1b was detected between cMo to erythroid progenitors in littermate controls, but fully

absent in *Ifnar1^{fl/fl} LysM^{cre/+}* animals. Further, myeloid cells derived chemokines like Ccl2, Ccl5, Cxcl9, and Cxcl10 can bind to erythroblasts via the atypical chemokine receptor 1 (ACKR1). The interaction of derived Ccl2 with Ackr1 was abolished in the absence of myeloid IFNAR, further illustrating the broad impact of myeloid IFNAR signaling on erythroblastic cytokine sensing. We also report that predicted cell-cell Vcam1 with erythroid Itga4, Itgb1, and Itgb was only present between intMo and the erythroid lineage was only significant in littermate controls. Thus, we found an overall reduced interaction between IFNAR deficient myeloid cells and erythroblasts, compared to controls, suggesting that activation of myeloid cells.

We sought to verify our findings and used FACS to isolate cMo (CD11b⁺ Ly-6C⁺) and RPMs (CD11b⁻ Ly-6C⁻ F4/80⁺) for RNA-sequencing at baseline and on days 1, 3, and 6 after CpG-ODN challenge in *Ifnar1^{fl/fl} LysM^{cre/+}* and littermate control spleens (Figure S5D). Principal component analysis (PCA) confirmed a strong transcriptional activation in cMo and RPMs on D1, but while monocytes almost returned to a baseline state by D6, RPMs displayed more persistent transcriptional alteration which was also reflected in the number of differentially expressed genes to baseline and between genotypes (Figure S5F and S5G). At the transcript level, macrophage markers and iron metabolism genes were downregulated in sorted monocytes from IFNAR-deficient mice (Figure S5I). In contrast, RPMs primarily exhibited reduced expression of type I IFN-associated genes, while iron metabolism programs remained largely preserved (Figure S5J). This suggests that mature RPMs are comparatively resilient to disrupted IFN sensing, whereas type I IFN signaling is critical for monocytes to acquire an RPM-like phenotype. As RPM numbers were unexpectedly increased in *Ifnar1^{fl/fl} LysM^{cre/+}* spleens on day 6 (Figure 2D), we speculated that these cells may be functionally impaired. GDF15 is an important regulator of niche signaling and RPM development, and was reduced in intMo in the scRNA-seq data and in sorted RPMs in the bulk RNA-seq data on day 1⁴¹. Reduced GDF15 transcription was even visible in whole-spleen homogenates showing significantly reduced Gdf15 mRNA levels in *Ifnar1^{fl/fl} LysM^{cre/+}* mice on days 1 and 3 after CpG challenge (Figure S5H). Interestingly, flow cytometric assessment of maturation markers for resident macrophages confirmed a maturation delay/defect in RPMs from *Ifnar1^{fl/fl} LysM^{cre/+}* mice, initially displaying higher F4/80 levels, followed by reduced F4/80, VCAM1 and CD64 expression on day 6. Collectively, these data suggest that type I IFN sensing regulates monocyte-derived RPM development, and that its loss results in delayed or incomplete maturation of monocyte-derived RPMs.

DISCUSSION

We show that myeloid type I IFN sensing amplifies splenic stress erythropoiesis. Upon CpG challenge, both erythroid progenitors and myeloid niche cells mounted a broad stress response, but erythroblast expansion was markedly reduced when IFNAR signaling was

selectively disrupted in myeloid cells. Time-resolved single-cell profiling revealed that myeloid IFNAR was required to sustain stress programs in splenic erythroid progenitors and to promote the acquisition of RPM-like features in recruited monocytes.

Our initial goal was to characterize key differences between extramedullary steady state- and stress erythropoiesis. However, transcriptional comparison of erythropoietic niche cells at steady state and at the peak of splenic erythroblast expansion revealed that the core erythroid differentiation program was largely preserved. However, our analysis still detected residual IFN-stimulated gene expression in erythroid progenitors and myeloid niche cells on day 6 after CpG challenge. We therefore asked whether CpG-induced type I IFN signaling contributes to the regulation of splenic stress erythropoiesis.

Splenomegaly of *Ifnar1^{fl/fl} LysM^{cre/+}* mice was markedly reduced and this could be largely attributed to reduced erythroblast expansion on day 6. However, we found that recruited monocytes fail to effectively activate RPM transcriptional programs compared to *Ifnar1^{fl/fl} LysM^{+/+}* controls already by day 1. Monocyte acquisition of iron-recycling red pulp macrophage phenotypes depends on the transcription factor SPI-C which was transcriptional downregulated in *Ifnar1^{fl/fl} LysM^{cre/+}* monocytes. Reduced *Spic* expression coincided with decreased *Gdf15* expression in intermediate monocytes and whole-spleen homogenates. Although *Gdf15* is broadly implicated in stress responses, it has been shown to play a critical role in inflammation-driven stress erythropoiesis and RPM development^{8,41,42}. SPI-C activation is driven by heme-dependent degradation of its suppressor BACH1 but can also incorporate TLR–MyD88 and IFN signaling^{8,13,14}. In TLR–MyD88 dependent models of macrophage SPI-C activation, IFN- γ has been described as a SPI-C repressor that shifts macrophages toward an inflammatory polarization state⁴³. In anemia models of chronic TLR7 or TLR9 activation *Spic* expression is induced in monocyte-derived macrophages, but this generates a hemophagocytic state rather than fully differentiated RPMs⁴⁴. This is in line with our observation that splenic RPM numbers were comparable to controls, but that *Ifnar1^{fl/fl} LysM^{cre/+}* RPMs displayed a less mature phenotype. We therefore propose that type I IFN sensing in monocytes integrates with heme- and TLR–MyD88-dependent cues to license iron-recycling programs and counteract type II IFN-driven inflammatory polarization.

In contrast to the myeloid compartment, the erythroid transcriptional response was comparable between genotypes on day 1. Surprisingly, we found consistent transcriptional activation of *Myd88* which is typically induced by TLRs or IL-1 family cytokines. It has been reported that mouse and human erythroblast express TLR9 and bind free CpG-containing DNA which increased erythrophagocytosis and IFN production by myeloid cells *in vivo*^{10,12}. While we did not detect *Tlr9* transcripts in the erythroid lineage the endosomal trafficking protein *Unc93b1*

was expressed at the MPP/MEP stage. It is therefore intriguing to speculate that stress BFU-Es may be directly stimulated by TLR9 activation. A central finding of our analysis was the detection of an IFN-response signature in erythroid progenitors at day 1, which was attenuated in *Ifnar1^{fl/fl}* *LysM^{cre/+}* by day 3. This was accompanied by reduced expression of the STAT family transcription factors *Stat1*, *Stat2*, and *Stat3*. STAT1 and STAT3 are not only activated by inflammatory cytokines but also activated downstream of EPOR signaling and are important regulators of erythroid development^{45–47}. Interestingly, attenuated IFN signaling was associated with increased expression of genes linked to EPO-responsive erythroid activation (*Irs2*, *Podxl*, *Cish*) and systemic iron mobilization (*Erfe*) at erythroblast stages. These data are consistent with a model in which IFN sensing at the CFU-E stage restrains terminal erythroid differentiation but reduced IFN signaling in the erythroid niche may relieve this constraint and shift JAK–STAT activity from IFN-associated signaling toward EPOR-JAK-STAT5 erythroid terminal differentiation.

Type I IFNs exert pleiotropic effects that are essential for host defense against diverse pathogens, but their dysregulated or sustained activation can also drive immunopathology⁴⁸. During induction of stress erythropoiesis pathways, type I IFN signaling may contribute to the development of monocyte derived RPMs and cytokine driven expansion of stress erythroid progenitors. However, in chronic inflammatory settings, sustained type I IFN signaling can contribute to anemia of inflammation by reinforcing IL6-dependent hepatic hepcidin (*Hamp*) induction, thereby suppressing ferroportin (*Slc40a1*), limiting macrophage iron export and promoting reticuloendothelial iron sequestration^{17,49,50}. Collectively, our data suggests that type I IFN signaling acts as a temporal regulator of stress erythropoiesis, supporting early inflammatory niche adaptation while imposing constraints on erythroid terminal differentiation to effectively expand stress progenitors. This dual role may help reconcile the beneficial effects of transient IFN signaling during acute stress responses with its pathogenic contribution to anemia under chronic inflammatory conditions.

MATERIAL AND METHODS

Mice and CpG-induced stress erythropoiesis model

All experiments in this study were performed in accordance with the ethical guidelines of the medical University of Vienna and the Austrian Ministry of Science (GZ: 66.009/0345-V/3b/2019). 9-12-week-old, age-matched, female C57BL6/J, were initially ordered from Janvier Labs. *IFNAR^{fl/fl}* *LysMCre^{+/+}* (herein referred to as IFNAR^{fl/fl} controls) and *IFNAR^{fl/fl}* *LysMCre^{cre/+}* (lacking Typ I IFN sensing in myeloid cells) littermates^{51,52} were obtained by crossing *IFNAR^{fl/fl}* females with *IFNAR^{fl/fl}* *LysMCre^{cre/+}* males. All mice were then bred in-house at the Medical University of Vienna, Core Facility for Laboratory Animal Breeding and

Husbandry in specific pathogen-free (SPOF) conditions and in accordance with FELASA (Federation of European Laboratory Animal Science Association) guidelines. All animals were fed chow diet ad libitum and kept under standardized conditions. Extramedullary stress erythropoiesis was induced as previously described⁵³, by injecting 10 nmol CpG-ODN 1826 intra peritoneally (i.p.) in a volume of 200µL PBS.

Sample preparation single-cell RNA-sequencing

To achieve a reasonable sequencing depth of wild type splenic cells at steady state and at the peak of stress induced erythropoiesis (Figure 1), spleens were harvested from untreated mice or 6 days post CpG challenge. Two spleens were pooled per condition. Single cell suspensions were prepared by squeezing spleens through a 70µm strainer, followed by lysis of mature erythrocytes. After staining with surface antibodies (CD19, B220, CD3, NK1.1, CD11b, Ly-6G, Ly-6C, CD45, CD11c, F4/80, cKit, Sca-1) we then enriched for myeloid-, erythroid- and stem cells, by selectively sorting a myeloid CD45+ fraction, negative for CD19, B220, CD3, Ly-6G and NK1.1, thereby removing the majority of B-, T-, NK cells and neutrophils. From the same cell suspension, we sorted lineage negative (CD19, B220, CD3, Ly-6G, NK1.1, CD11b, F4/80) progenitors and stem cells. The myeloid and progenitor fraction was pooled, and cells were subjected to single cell sequencing. For comparing *IFNAR*^{fl/fl} control and *IFNAR*^{fl/fl} *LysMCre*^{+/-} animals, we performed aforementioned procedure but cells were hashed with barcoding antibodies (TotalSeq™-C Antibodies, C01-C06, Biolegend) for multiplex single cell sequencing.

Bulk RNA-sequencing analysis of sorted cells

Erythroblasts (Lin-, Ter-119+) and RPM (CD45+, Lin-, Ter-119-) were sorted from *IFNAR*^{fl/fl} control and *IFNAR*^{fl/fl} *LysMCre*^{+/-} spleens at indicated timepoints and subjected to bulk RNA sequencing. Differential expression analysis was performed with DESeq2 (v1.46.0). Genes were filtered per cell type to retain those with at least 10 counts in three samples of at least one sample group, pairwise comparisons were performed using Wald tests, and response genes were additionally required to pass a likelihood ratio test. P-values were adjusted using independent hypothesis weighting⁵⁴ (v.1.34.0), with mean gene expression as a covariate, controlling the false discovery rate at $\alpha=0.05$.

General single-cell RNA-sequencing data preprocessing

VBC sequencing data were demultiplexed and converted to FASTQ with Illumina bcl2fastq (v2.20.0) by the sequencing facility. Reads were aligned to the 10x Genomics mouse mm10-2020-A reference build. Count matrices were created using Cell Ranger (v9.0.1) with the count function. Demultiplexing of sample source feature barcodes was performed using DropletUtils

(v1.26.0) with minimum threshold for log-fold change of 0.5. The Cell Ranger filtered count matrices were imported and subset by percentage mitochondrial reads ($\leq 5\%$), unique molecular identifiers (UMIs) per cell (≥ 1000) and detected gene per cell (≥ 6000). Doublets were identified per sequencing facility dataset using the SOLO⁵⁵ implementation in scvi-tools⁵⁶ (v1.2.2) and removed using a doublet probability threshold of ≥ 0.5 . The data were stored as Seurat (v5.2.1) or AnnData (v0.11.4) object for compatibility with R (v4.4.1) and Python (v3.10.16) respectively.

Data integration and dimensional reduction

We employed scVI-tools single cell variational inference (scVI)⁵⁶ for data harmonization. All models were configured with a 30-dimensional latent space, two hidden layers with 128 nodes each, a negative binomial gene likelihood distribution and the sample identifier as batch key. Further we informed the model with continuous covariates about biological (S-phase score, G2M-phase score and technical variance (sequencing facility identifier)). The feature space was restricted to genes with at least three counts in at least three cells. The model was trained for 157 epochs following the heuristic implemented in scvi-tools. The latent space of the trained model was used to compute k-nearest neighbors (KNN) graph (30 neighbors), Leiden clustering (resolution 1), and uniform manifold approximation and projection (UMAP) with Scanpy (v1.11.1)

Cell type annotation

Leiden clusters were annotated based on published marker gene lists and further supported by automated annotation using reference bulk RNA-sequencing data from Haemopedia⁵⁷ and ImmGen⁵⁸ with SingleR⁵⁹ (v2.8.0). Cluster with conflicting marker expression were further split for high resolution annotation (resolution 0.5). If the expression pattern strongly implied a cell type mixture, the respective cluster was removed from downstream analysis. The annotation yields the final set of 46.765 high quality cells that were used for downstream analysis.

Differential expression analysis

For each group comparison within a cell type cluster, genes were filtered to retain those expressed in $\geq 5\%$ of cells and with ≥ 3 counts in at least 3 cells. A minimum of 3 cells per group was required to perform the Wilcoxon test using the Seurat Presto implementation. P-values were adjusted using independent hypothesis weighting (v.1.34.0), with mean gene expression as a covariate, controlling the false discovery rate at $\alpha=0.05$.

Diffusion pseudotime

For trajectory inference, spliced and unspliced count matrices were generated with Velocityto (v0.17.17), and CellRank^{60,61} (v2.0.7) was used to construct a transition matrix from an RNA-velocity kernel based on the dynamical model of transcriptome kinetics. To increase robustness, this kernel was combined with a connectivity kernel derived from the scVI latent space using equal weights. Macrostates were identified by generalized Perron cluster cluster analysis (GPCCA), and the MegP and erythroblast populations were specified as terminal states. Fate probabilities toward these terminal states were then computed for each cell. A neighborhood graph was recomputed on the subset of MEP, CFU-E, and erythroblast cells using the scVI latent representation, and diffusion pseudotime (DPT) was computed using Scanpy, with the root cell defined as the MEP cell exhibiting the lowest fate probability toward the erythroblast terminal state.

Pseudotime-associated genes

To identify genes with differential expression patterns along erythroid trajectories, we applied tradeSeq²² (v1.13.12), which models gene expression as a function of pseudotime using negative binomial generalized additive models (NB-GAMs). In tradeSeq, gene expression is modeled using cubic smoothing splines, whose flexibility is determined by the placement of knots along pseudotime. By default, knots are positioned at quantiles of the pseudotime distribution, which can bias the fit toward densely populated regions. To mitigate this effect given compositional shifts toward erythroblast states, we defined knot locations based on the median pseudotime of each cell type (MEP, CFU-E, and erythroblasts) and the boundary values (0 and 1). This strategy ensured balanced representation of all differentiation stages. The model was then fitted for each gene, and pseudotime-associated genes were identified using association test with consecutive and end contrasts (adj. p-value ≤ 0.05). Genes were considered pseudotime-associated if they reached significance in either test.

Trajectory-based differential expression analysis

To assess differences between conditions, we applied the tradeSeq condition test to all pseudotime-associated genes. Genes with a Benjamini-Hochberg-adjusted p-value ≤ 0.05 from the Wald test were classified as perturbed. Perturbed genes were classified as upregulated or downregulated across pseudotime using a bin-based subsampling approach. Cells were divided into ten equally sized pseudotime bins, each condition was subsampled to the lowest cell number per bin for 500 iterations, and differential expression was tested on normalized counts using a Wilcoxon rank-sum test. Directionality was assigned only if at least 80% of significant tests within a bin showed the same log2FC sign, this sign matched the smoothed bin-level log2FC, and all significant bin-level log2FC values for the gene showed the same direction.

Gene regulatory network analysis (GRN)

For the GRN analysis we used pycscenic⁶² (v0.12.1) with the GRNBoost2 algorithm and followed the Nature Protocol⁶³.

Cell-cell interaction (CCI) analysis

Genes were retained if they had ≥ 3 counts in at least three cells, and one CellChat^{64,65} (v2.2.0) object was generated per sample group. Genes were not filtered based on overexpression testing, and communication probabilities were computed with population-size weighting using a truncated mean expression estimator with a trim value of 0.01.

Differential abundance testing

We constructed the miloR⁶⁶ (v2.2.0) graph using the scVI latent space. A neighborhood parameter of $k=30$ was selected, resulting in an average neighborhood size of approximately x cells. Neighborhoods were tested for group enrichment, and a false discovery rate (FDR) correction was performed using graph-overlap weighting.

Gene set enrichment analysis (GSEA)

For group comparisons, genes were ranked by multiplying the signed log2FC by the negative log10-adjusted P value. Gene set enrichment analysis was performed with fgsea (v1.32.4) using MSigDB⁶⁷ mouse Gene Ontology biological process gene sets obtained through msigdb (v25.1.1).

Plotting and visualization

Plots were generated in R using ggplot2 (v3.5.2) and ComplexHeatmap (v2.22.0), and final figure panels were assembled and formatted in Adobe Illustrator.

Figures

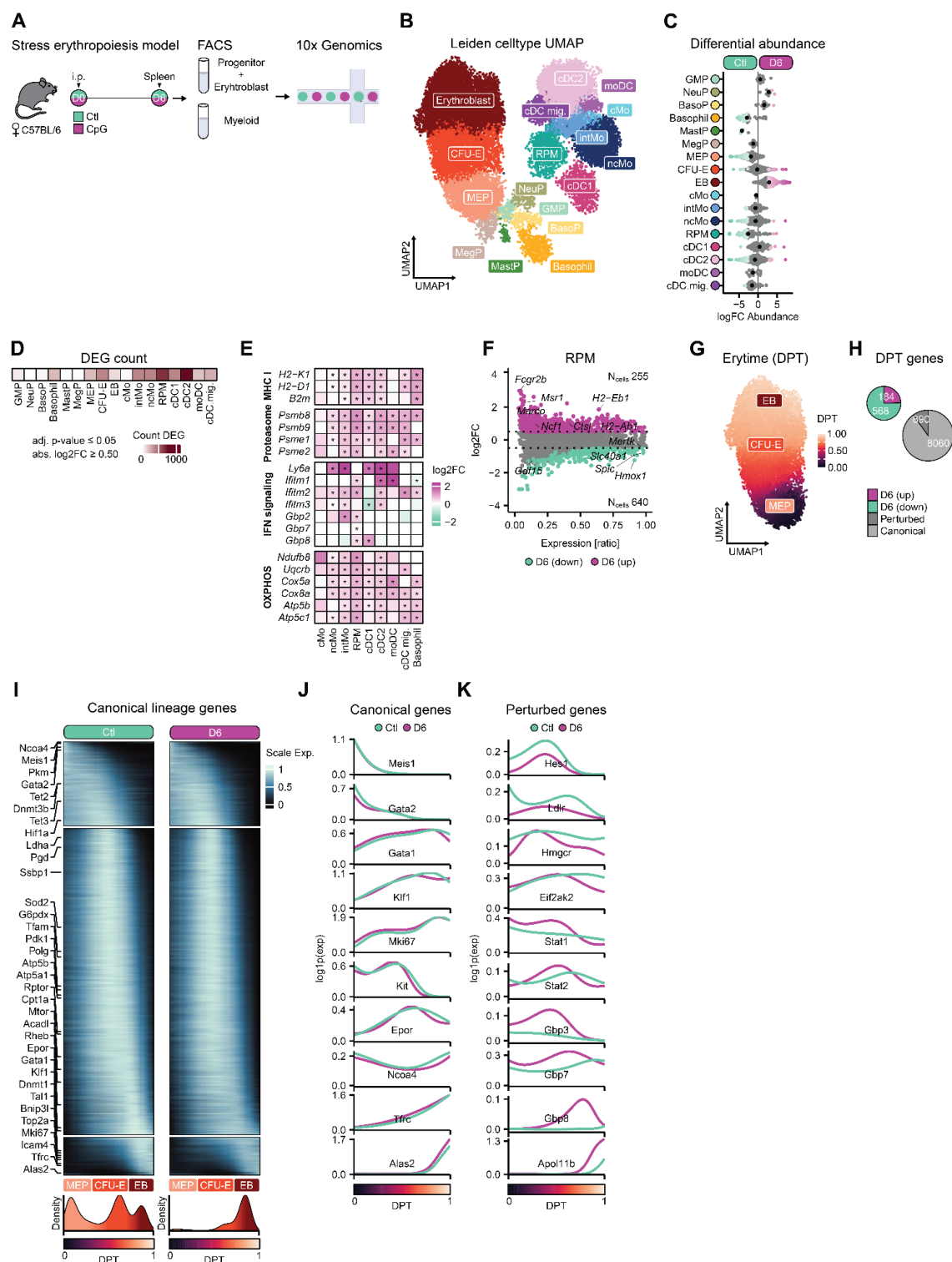


Figure 1 Stress erythropoiesis activates an interferon-responsive gene program. (A)

Experimental setup for scRNA-seq analysis of sorted splenic progenitor/erythroid and myeloid cells from PBS-treated control and CpG-ODN-challenged mice on day 6. **(B)** UMAP embedding of integrated single-cell transcriptomes annotated by Leiden cluster-based cell type assignment. **(C)** Differential abundance analysis across graph neighborhoods comparing control and day 6 CpG-treated spleens. Dots represent neighborhoods; color indicates significant enrichment in either condition (adj. p-value ≤ 0.05). **(D)** Heatmap showing the number of differentially expressed genes per cell type between control and day 6 CpG-treated spleens using Wilcoxon test (adj. p-value ≤ 0.05 and absolute log2 fold change ≥ 0.5). **(E)** Heatmap showing log2 fold changes of selected genes across annotated cell types. Asterisks indicate significant differential expression. **(F)** Dot plot showing differentially

expressed genes in red pulp macrophages, with log2 fold change plotted against expression ratio. Significant upregulated and downregulated genes are colored by condition. **(G)** UMAP embedding of erythroid lineage cells colored by diffusion pseudotime (DPT). **(H)** Pie chart summarizing trajectory-based differential expression analysis of DPT-associated genes, classified as canonical or perturbed between control and day 6 CpG-induced stress erythropoiesis. **(I)** Heatmap showing scaled expression of canonical erythroid lineage genes over pseudotime in control and day 6 CpG-treated cells. Genes are grouped according to their expression maximum at the MEP, CFU-E or erythroblast stage; density plots below indicate cell type distribution along pseudotime. **(J)** Smoothed expression of selected canonical erythroid genes along pseudotime in control and day 6 CpG-treated cells. **(K)** Smoothed expression of selected perturbed genes along pseudotime in control and day 6 CpG-treated cells.

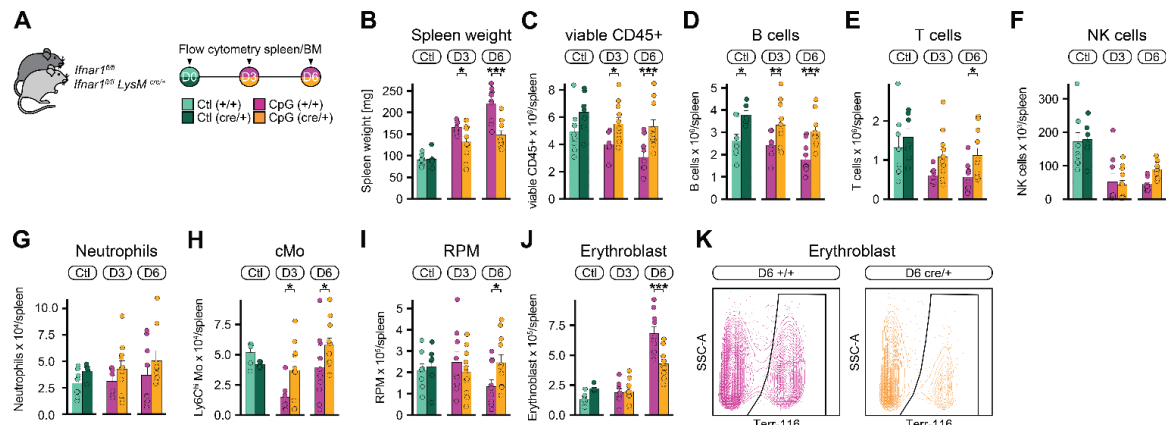


Figure 2 Myeloid Type I IFN sensing boosts extramedullary erythroblast expansion.

(A) Experimental setup for flow cytometric analysis of spleen and bone marrow from *Ifnar1^{fl/fl}* *LysM^{+/+}* control mice and *Ifnar1^{fl/fl}* *LysM^{cre/+}* mice at baseline and on days 3 and 6 after CpG-ODN challenge. **(B)** Spleen weight at baseline and after CpG-ODN challenge. **(C)** Number of viable CD45⁺ leukocytes per spleen. **(D–J)** Absolute numbers of splenic B cells (**D**), T cells (**E**) NK cells (**F**) neutrophils (**G**) Ly6C^{hi} monocytes (**H**) red pulp macrophages (**I**) and erythroblasts (**J**) per spleen. Statistical significance was assessed by two-way ANOVA followed and post hoc Sidak-adjusted pairwise genotype comparison. Results are indicated with *P < 0.05, **P < 0.01, ***P < 0.001. **(K)** Representative flow cytometry plots showing erythroblast maturation distribution in *Ifnar1^{fl/fl}* *LysM^{+/+}* control and *Ifnar1^{fl/fl}* *LysM^{cre/+}* mice on day 6 after CpG-ODN challenge.

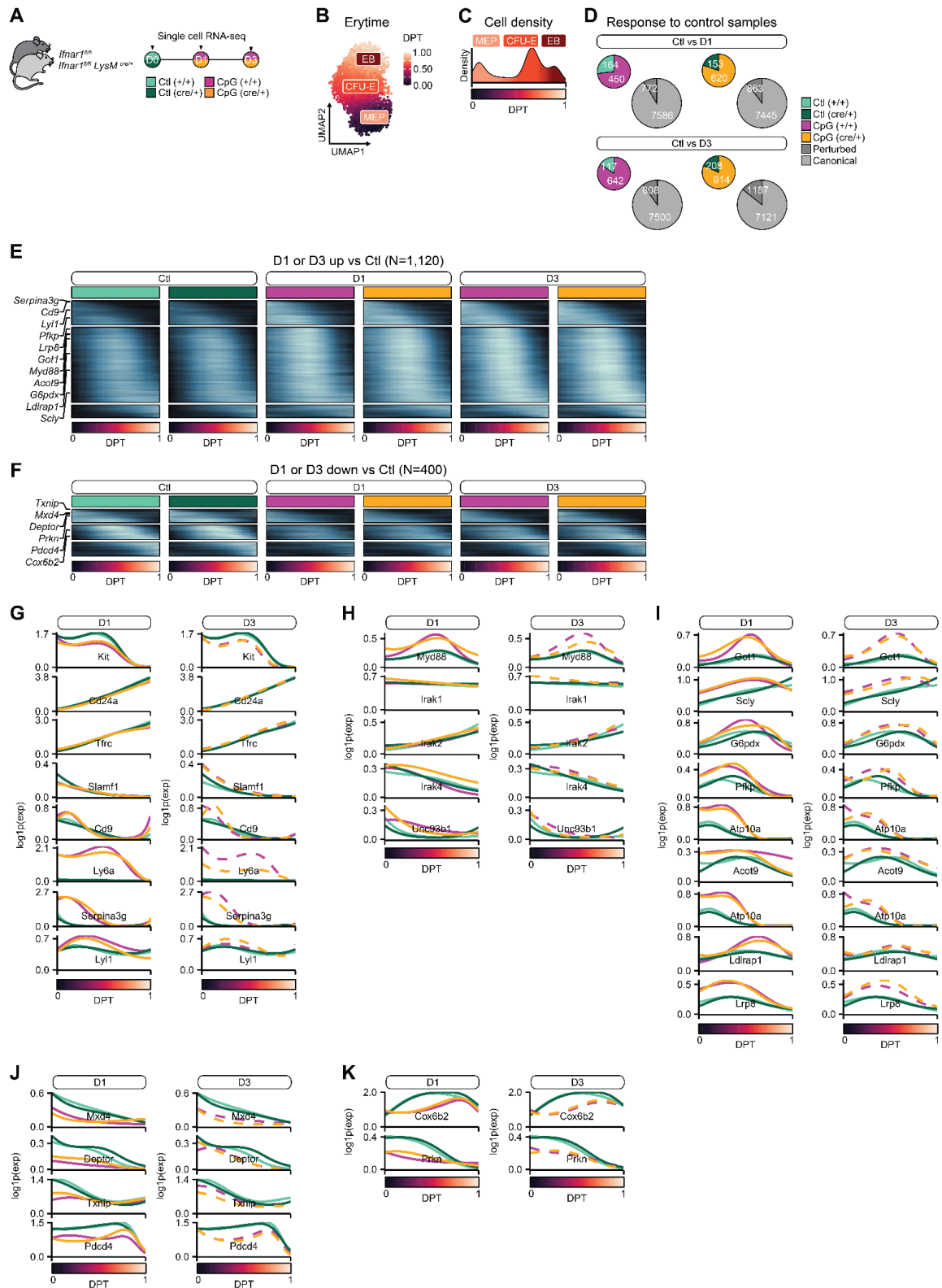


Figure 3 Unaffected erythroid MyD88 responses and metabolic reprogramming in myeloid IFNAR deficiency.

(A) Experimental setup for scRNA-seq analysis of sorted splenic cells from *Ifnar1^{fl/fl}* *LysM^{cre/+}* control mice and *Ifnar1^{fl/fl}* *LysM^{cre/+}* mice at baseline and on days 1 and 3 after CpG-ODN challenge. **(B)** UMAP embedding of erythroid lineage cells colored by diffusion pseudotime (DPT) and annotated as MEP, CFU-E and erythroblast (EB). **(C)** Density distribution of MEPs, CFU-Es and erythroblasts along erythroid pseudotime of pooled samples. **(D)** Pie charts summarizing trajectory-based differential expression analysis comparing day 1 or day 3 after CpG-ODN challenge with control samples in each genotype. Genes are classified as canonical, perturbed or condition responsive. **(E,F)** Heatmaps showing smoothed expression dynamics over erythroid pseudotime for genes

upregulated (**E**) or downregulated (**F**) on day 1 or day 3 after CpG-ODN challenge compared with control samples. Selected genes are indicated. (**G-H**) Smoothed expression of selected genes along pseudotime on days 1 and 3 after CpG-ODN challenge, highlighting genes upregulated or downregulated relative to baseline in both genotypes.

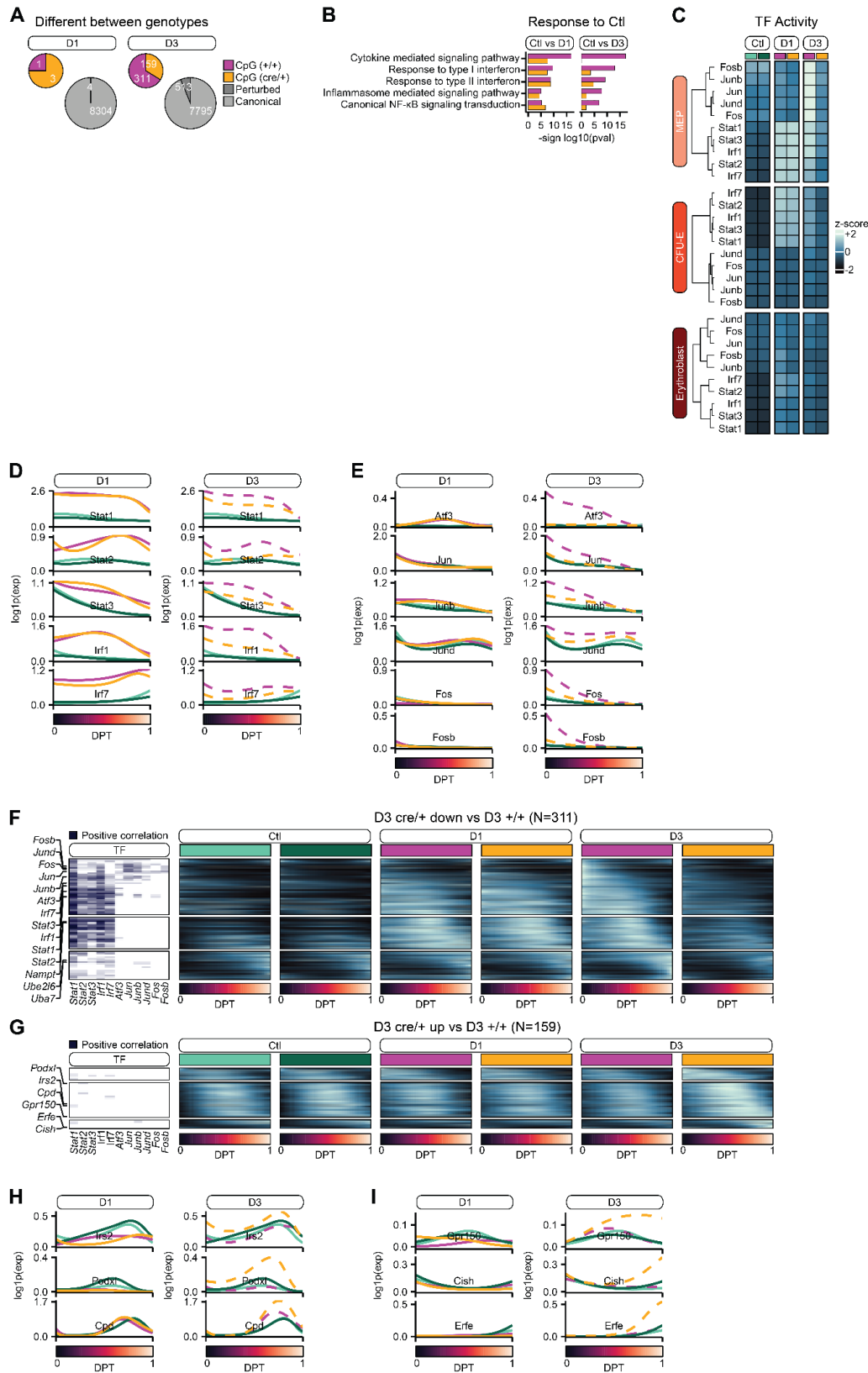


Figure 4 Sustained interferon and AP-1 transcriptional programs in erythroid progenitors require myeloid IFNAR signaling.

(A) Pie charts summarizing trajectory-based differential expression analysis comparing *Ifnar1^{fl/fl}* *LysM^{cre/+}* and *Ifnar1^{fl/fl}* *LysM^{+/+}* erythroid cells on days 1 and 3 after CpG-ODN challenge. (B) Gene set enrichment analysis of genes upregulated in erythroid cells on days 1 and 3 after CpG-ODN challenge compared with control samples. Bars show $-\log_{10}$ -transformed P values for selected pathways for each genotype. (C) Heatmap showing

hierarchical clustering of scaled transcription factor activity scores across MEPs, CFU-Es and erythroblasts in control and CpG-treated mice. **(D,E)** Smoothed expression of selected STAT/IRF module genes **(D)** and AP-1 module genes **(E)** along erythroid pseudotime on days 1 and 3 after CpG-ODN challenge. **(F,G)** Heatmaps showing smoothed expression dynamics of genes downregulated **(F)** or upregulated **(G)** in *Ifnar1^{fl/fl}* *LysM^{cre/+}* compared with *Ifnar1^{fl/fl}* *LysM^{+/+}* erythroid cells on day 3 after CpG-ODN challenge. Left panels indicate positive correlations between genes and selected transcription factor regulons. **(H,I)** Smoothed expression of selected genes upregulated in *Ifnar1^{fl/fl}* *LysM^{cre/+}* erythroid lineage cells on day 3 after CpG-ODN challenge.

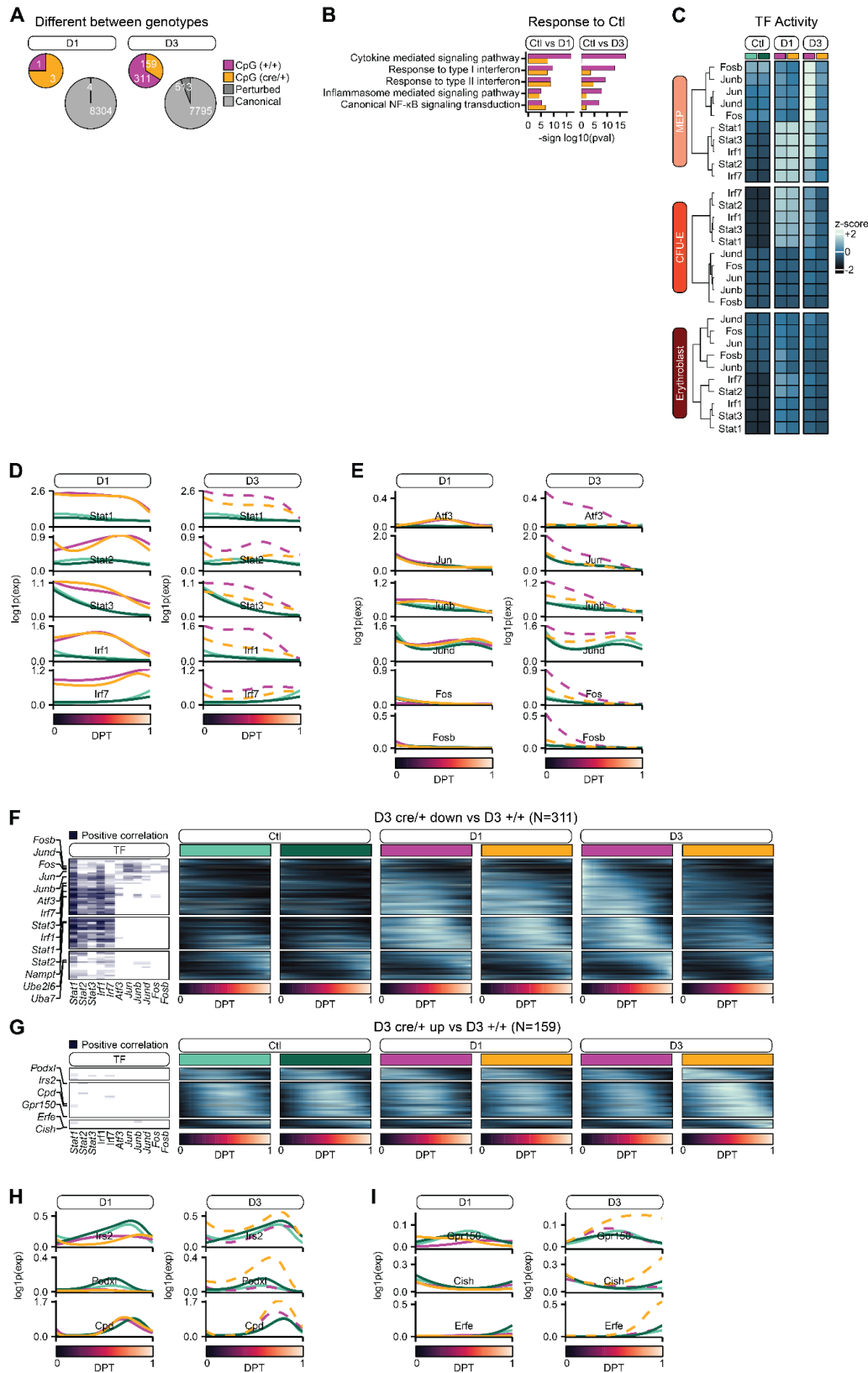


Figure 5 Type I interferon sensing supports tissue adaptation of monocyte derived RPMs.

(A) Heatmap showing the number of differentially expressed genes per cell type on days 1 and 3 after CpG-ODN challenge compared with control samples. (B) Heatmap showing the number of differentially expressed genes per cell type between *Ifnar1^{fl/fl} LysM^{cre/+}* and *Ifnar1^{fl/fl} LysM^{+/+}* mice at baseline and on days 1 and 3 after CpG-ODN challenge. (C) Gene regulatory network analysis of intermediate monocytes showing top 10 transcription factors with differential activity between genotypes on days 1 and 3 after CpG-ODN challenge. (D) Inferred Spic transcription factor activity in intermediate monocytes across genotypes and time points. (E) Gene set enrichment

analysis of intermediate monocytes showing selected top pathways differentially regulated between genotypes on days 1 and 3 after CpG-ODN challenge. **(F,G)** Heatmaps showing log2 fold changes and scaled expression of selected interferon-response, myeloid activation, macrophage adaptation and inflammatory genes in intermediate monocytes **(F)** and classical monocytes **(G)** across genotypes and time points. Asterisks indicate significant differential expression. **(H)** Circos plots showing predicted cytokine and chemokine communication from classical and intermediate monocytes to erythroid progenitors and erythroblasts on days 1 and 3 after CpG-ODN challenge in *Ifnar1^{fl/fl}* *LysM^{+/+}* and *Ifnar1^{fl/fl}* *LysM^{cre/+}* mice.

Supplementary figures

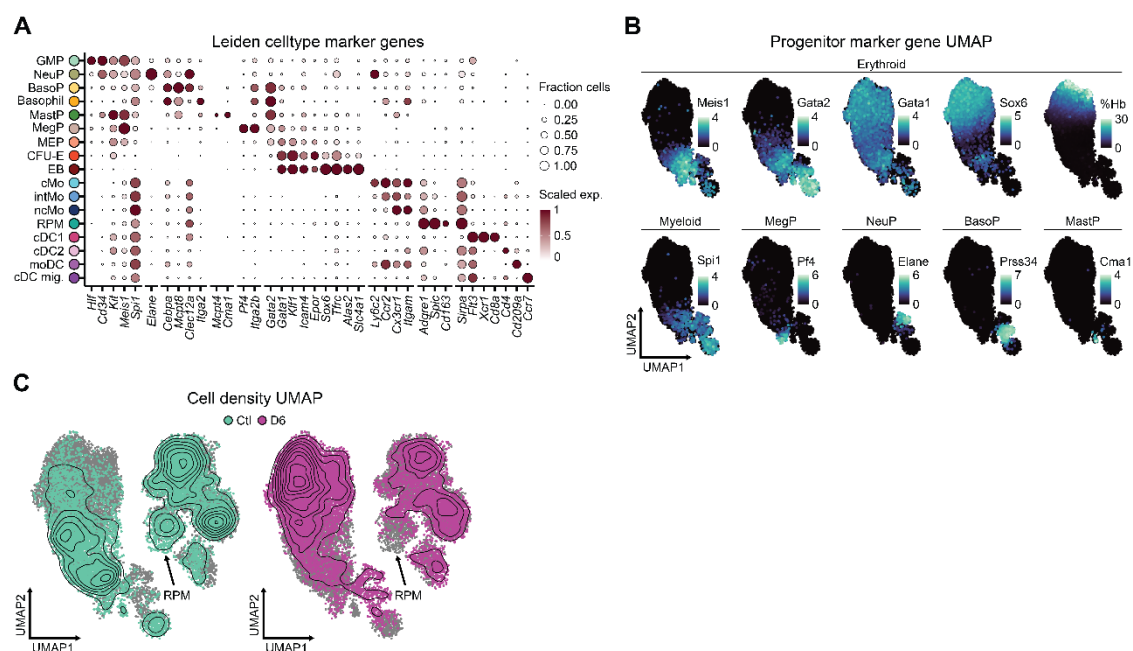


Figure 1

(A) Dot plot showing expression of selected marker genes across annotated Leiden cell type clusters. Dot size indicates the fraction of cells expressing each gene, and color indicates scaled expression. (B) UMAP embeddings showing log normalized expression of selected marker genes used to support cell type annotation. Hemoglobin is scored in percentage reads per cell total UMI (C) Cell density UMAPs showing the distribution of control and day 6 CpG-treated cells across the integrated embedding. Cells not belonging to the indicated group are shown as a grey background. Arrows highlight the red pulp macrophage region.



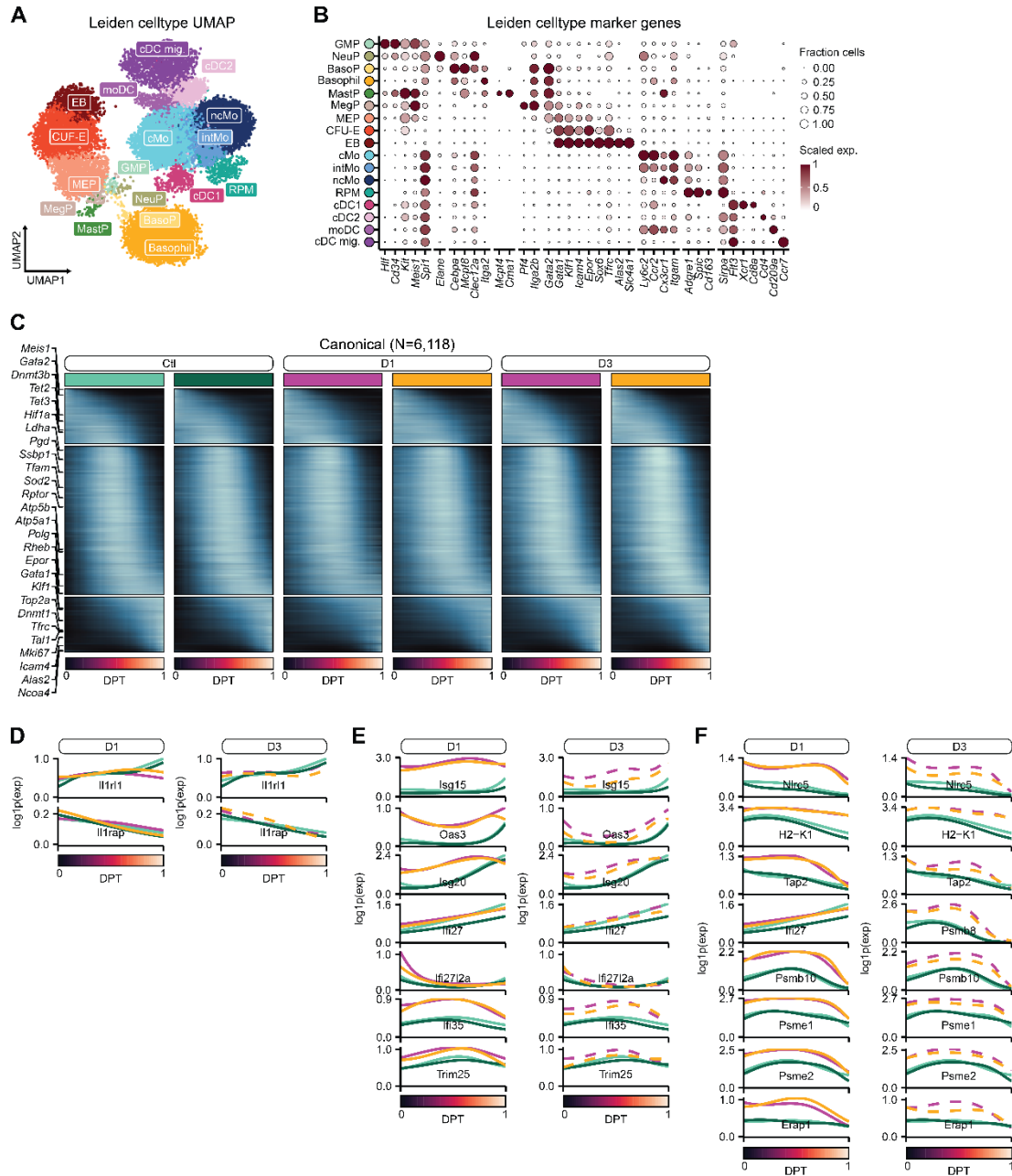


Figure 3

(A) UMAP embedding of integrated single-cell transcriptomes from *c* control and *Ifnar1^{fl/fl} LysM^{cre/+}* mice time-course scRNA-seq dataset, annotated by Leiden cluster-based cell type assignment. (B) Dot plot showing expression of selected marker genes across annotated Leiden cell type clusters. Dot size indicates the fraction of cells expressing each gene, and color indicates scaled expression. (C) Heatmap showing smoothed expression dynamics of canonical erythroid lineage genes along diffusion pseudotime in control, day 1 and day 3 CpG-treated samples per genotype. (D–F) Smoothed expression of selected genes along erythroid pseudotime on days 1 and 3 after CpG-ODN challenge.

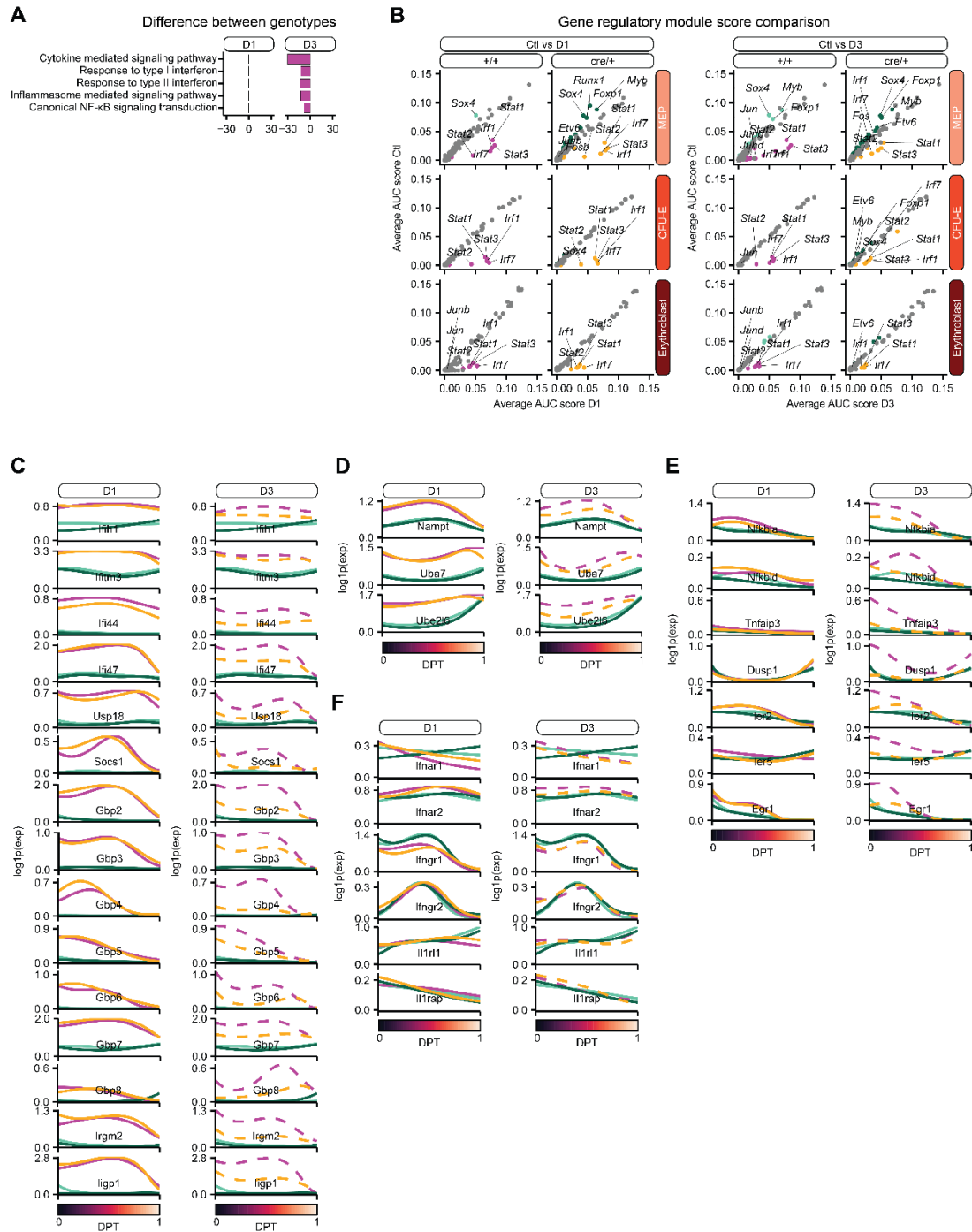


Figure 4

(A) Gene set enrichment analysis of genes differentially expressed between *Ifnar1^{fl/fl} LysM^{+/+}* and *Ifnar1^{fl/fl} LysM^{cre/+}* erythroid cells on days 1 and 3 after CpG-ODN challenge. Bars show direction and strength of enrichment for selected pathways. (B) Scatter plots comparing average transcription factor regulon AUC scores between control and CpG-treated samples on days 1 and 3, separated by genotype and erythroid cell state. Selected transcription factors are labeled. (C–F) Smoothed expression of selected genes on days 1 and 3 after CpG-ODN challenge.

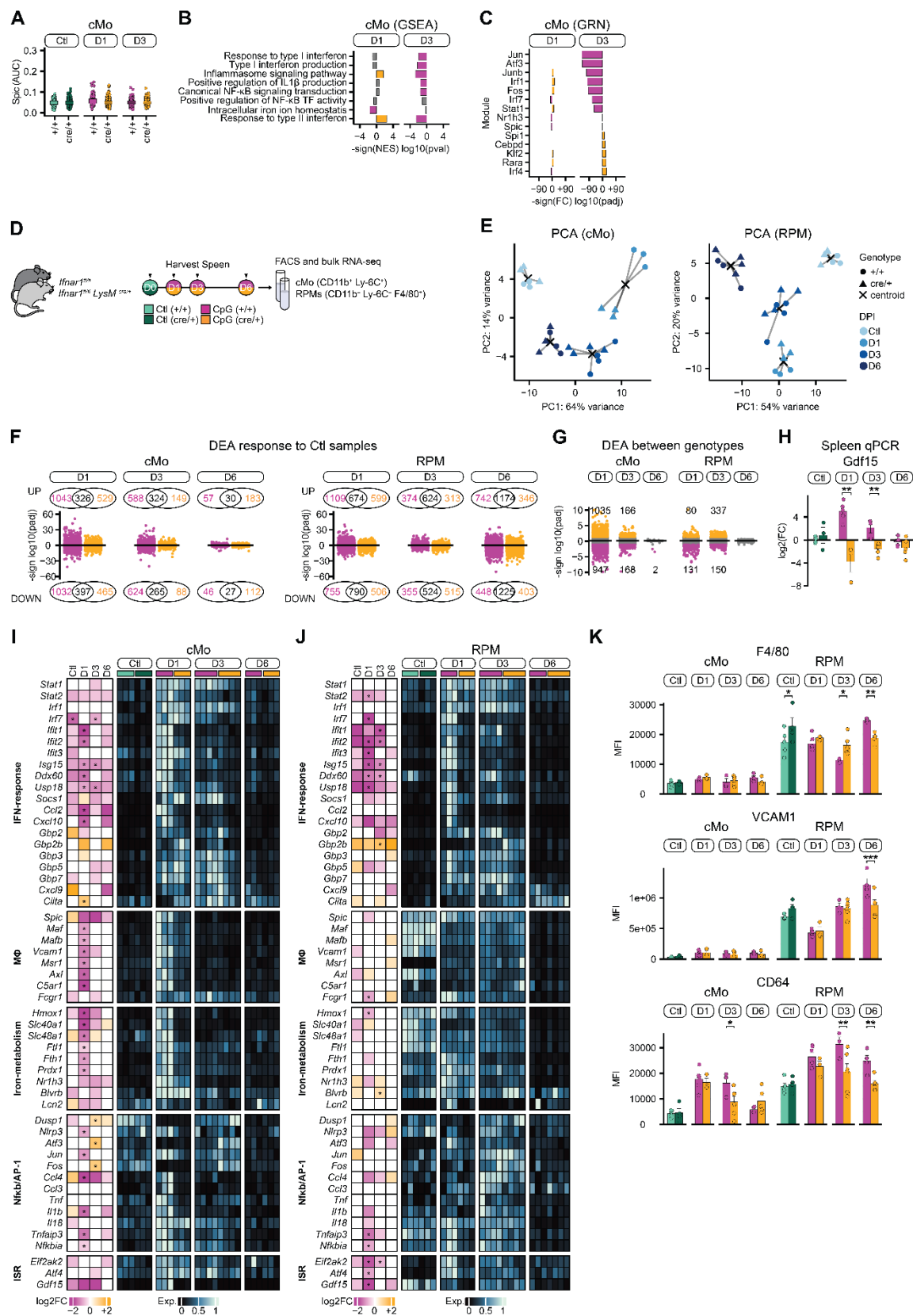


Figure 5

(A) Inferred Spic transcription factor activity in classical monocytes across genotypes and time points. **(B)** Gene set enrichment analysis of classical monocytes showing selected pathways differentially regulated between genotypes on days 1 and 3 after CpG-ODN challenge. **(C)** Gene regulatory network analysis of classical monocytes showing selected transcription factors with differential activity between genotypes on days 1 and 3 after CpG-ODN challenge. **(D)** Experimental setup for bulk RNA-seq analysis of sorted classical monocytes and red pulp macrophages from *Ifnar1^{fl/fl} LysM^{+/+}* control and *Ifnar1^{fl/fl} LysM^{cre/+}* mice at baseline and on days 1, 3 and 6 after CpG-ODN challenge. **(E)** Principal component analysis of bulk RNA-seq profiles from sorted classical monocytes and red pulp macrophages. **(F)** Differential expression analysis comparing CpG-treated samples with baseline controls in sorted classical monocytes and red pulp macrophages. Venn diagrams indicate the number of differentially expressed genes in each genotype and the overlap between genotypes. **(G)** Differential expression analysis comparing *Ifnar1^{fl/fl} LysM^{cre/+}* and *Ifnar1^{fl/fl} LysM^{+/+}* mice in sorted classical monocytes and red pulp macrophages at each time point. **(H)** qPCR analysis of *Gdf15* expression in whole-spleen homogenates at baseline and after CpG-ODN challenge. **(I, J)** Heatmaps showing log2 fold changes and expression of selected gene modules in sorted classical monocytes **(I)** and red pulp macrophages **(J)** across genotypes and time points. Asterisks indicate significant differential expression. **(K)** Flow cytometric quantification of F4/80, VCAM1 and CD64 mean fluorescence intensity in classical monocytes and red pulp macrophages across genotypes and time points.

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Author contributions: RG, SK and FD conceptualized the study. RG and SK supervised the study and provided funding. RG, ADG, AFa and FQ processed samples and prepared single cell and bulk RNA sequencing libraries. RG, AH, KL, AFa, LP, FM, and AFo performed in vivo validation and flow cytometry experiments. FD performed the bioinformatics analysis. FD prepared the figures. SK, RG, and FD wrote the manuscript.

Competing interest: The authors declare no competing interest.

Data and materials availability: All count matrices and raw sequencing data of single-cell and bulk RNA sequencing generated in this study will be made available upon publication in the NCBI gene Expression Omnibus database (GEO). Codes used for analyses performed in this manuscript, as well as the integrated single-cell data objects with UMAP and pseudotime will be made available on Zenodo.

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2.2 Disruption of skin homeostasis reveals divergent reconstitution dynamics of human skin tissue-resident memory T cell subsets

Title:

Disruption of skin homeostasis reveals divergent reconstitution dynamics of human skin tissue-resident memory T cell subsets

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ABSTRACT

Tissue-resident memory T cells (TDRM) regulate homeostasis and inflammation, yet their maintenance and functional adaptation in humans remain poorly understood. Here, we study dynamic changes in human skin TDRM in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT), with or without graft-versus-host disease (GVHD).

Using single-cell transcriptomic and protein-level profiling, we map host- and donor-derived TDRM over time. We identify CD8⁺ type 17-polarized TDRM (T17DRM) as a distinct subset, selectively depleted after transplantation, associated with a shift toward type 1-polarized T cells dominating the post-transplant TDRM compartment. Donor-derived T cells efficiently populate the skin but fail to reconstitute T17DRM, indicating a subset-specific impairment in persistence and generation. Delayed recovery of TDRM subset balance coincides with restoration of tissue homeostasis, implicating disturbed TDRM composition and function in post-transplant tissue dysregulation.

Together, these findings reveal dynamic remodeling of human skin TDRM and propose TDRM subset balance as key determinant of tissue homeostasis.

INTRODUCTION

Human skin harbors a dense network of immune cells, including tissue resident memory T cells. In line with recently proposed guidelines for T cell nomenclature¹, we refer to these cells as TD_RM. TD_RM are central to tissue homeostasis²⁻⁴, but also contribute to inflammation and the pathogenesis of skin diseases⁵⁻⁷, including acute cutaneous graft-versus-host disease (GVHD)⁸⁻¹⁰. GVHD is a T cell-mediated inflammatory condition following allogeneic hematopoietic stem cell transplantation (HSCT), with the skin representing a primary target organ¹¹. GVHD is predominantly induced by type 1-polarized, alloreactive donor T cells, causing damage in target tissues¹¹.

We and others have previously demonstrated the long-term persistence of host-derived TD_RM in the skin following myeloablative conditioning of HSCT recipients^{8-10,12}. Notably, host TD_RM are not fully replaced after donor engraftment but can persist for more than a decade after transplantation^{9,12}. These observations position HSCT as a unique human model for studying TD_RM reconstitution, maintenance and function in both steady-state conditions and T cell-mediated inflammation.

Skin TD_RM comprise developmentally and functionally different subsets. In murine models, most studies have focused on type 1 (T₁)-polarized T₁DRM, which arise in response to viral infection and depend on transcription factors Hobit and T-bet^{13,14}. In contrast, type 17 (T₁₇)-polarized T₁₇DRM develop independently, are driven by c-MAF and IL-7 signaling, express CCR6 and can be induced by commensal colonization, such as *Staphylococcus epidermidis*¹⁴⁻¹⁶. These cells are ICOS⁺ and can be selectively depleted by targeting ICOS¹⁴.

This has led to a conceptual distinction between type 17-polarized T cells – defined by shared functional and transcriptional features such as IL-17 production, ROR γ t and CCR6 expression – and T₁₇DRM, a resident subset with a distinct developmental trajectory, that in mice is characterized by CCR6, CD127 and c-MAF¹⁴. In human skin, CCR6⁺ T₁₇DRM-like cells are enriched in psoriasis^{6,17} and are functionally distinct from CD49a⁺ epidermal TD_RM¹⁸. CCR6⁺ T cells produce IL-17A, IL-22, TNF- α and GM-CSF, whereas CD49a⁺ T cells exhibit a T₁DRM-like phenotype and primarily produce IFN- γ ¹⁸. However, whether human TD_RM subsets are governed by transcriptional programs analogous to those described in mice (Hobit/IL-15 vs c-MAF/IL-7/ICOS) remains unclear.

To address this, we investigated TD_RM heterogeneity and dynamics in human skin following HSCT, leveraging samples from 85 transplant recipients with or without GVHD. We generated a high-dimensional, time-, tissue-, genotype- and clonotype-resolved single-cell RNA and T cell receptor (TCR) sequencing dataset comprising samples from 12 patients. This enabled

detailed analysis of T_{D_R}M composition, clonal relationships, and the tissue microenvironment, complemented by functional validation. We focused particularly on T₁₇D_RM-like phenotypes. Throughout, we use “T1” or “T17” to denote polarization states independent of residency, and “T₁D_RM” or “T₁₇D_RM” to describe skin-derived T cells exhibiting both polarization–associated as well as residency features based on transcriptional profiles and protein profiles.

We find that T₁₇D_RM are selectively depleted following transplantation and are not efficiently replenished, resulting in a transient skewing of the cutaneous immune landscape toward T1 responses. However, this imbalance resolves during recovery, highlighting the plasticity and adaptability of T_{D_R}M and implying that the equilibrium between T_{D_R}M subsets is critical for maintaining tissue homeostasis.

RESULTS

Single-cell transcriptomic profiling reveals dynamic immune chimerism and tissue-specific reconstitution after HSCT

To dissect developmental and temporal dynamics of human TDRM reconstitution *in vivo*, we performed single-cell RNA and TCR sequencing of paired skin and blood samples from HSCT recipients across four defined timepoints (Baseline (before myeloablative conditioning), day of transplantation (Tx), day 14 and day 100 post-transplantation (D14, D100)) (HSCT cohort, n = 7 patients, 21 samples, Fig. 1a,b). This was complemented by samples from five patients with acute cutaneous GVHD¹⁹, which were sampled at disease onset (Fig. 1a,b, GVHD). One patient (P6) was part of both cohorts. Detailed clinical characteristics of patients are listed in Supplementary Table S1.

After quality control and filtering, we recovered a total of 84,447 high-quality cells spanning immune and non-hematopoietic compartments (Fig. 1c, Extended Data Fig. 1a), capturing the major cellular constituents of human skin and blood (Fig. 1d,e) (see Methods and Supplementary Fig. S1). Within CD45⁺ lymphocytes, we detected 8 T cell and 2 NK cell clusters, alongside other lymphocyte, myeloid and non-hematopoietic cell populations across blood and skin (Fig. 1e,f, Supplementary Fig. S1a-g). Cell populations segregated primarily by tissue origin (Fig. 1d) with stable representation of most stromal populations across time, whereas immune compartments – particularly circulating T cells – underwent pronounced compositional perturbations especially within T cells in blood at D14 and during GVHD (Extended Data Fig. 1b-d). Collectively, we generated the first time-resolved HSCT single-cell RNA and TCR sequencing dataset of two tissue types, comprising the main immune and non-hematopoietic cell types of human blood and skin.

Using single nucleotide polymorphisms (SNPs) or gender mismatch enables tracking donor cell repopulation and host cell maintenance of the skin and blood over time^{9,12}. Employing SNP-based demultiplexing to assign cell genotypes²⁰ (Fig. 1g), we robustly resolved host and donor genotypes at single-cell resolution (Fig. 1h,i). Less than 0.5% of cells being falsely classified as donor cells, indicating high specificity of genotype assignment (Fig. 1h,i). In each host/donor pair we identified a set of unique discriminative SNPs (Fig. 1j). While blood immune cells were rapidly replaced by donor-derived cells, skin exhibited delayed and incomplete donor chimerism (Fig. 1k). Notably, immune cell diversity (richness) within the skin was higher during GVHD, suggesting dynamic remodeling of the tissue immune compartment (Fig. 1k).

Host cells made up ~79% of skin immune cells on D14 and ~17% on D100 and in GVHD (Fig. 1k).

In summary, these data establish a time-resolved framework to track human TD_{RM} persistence and replacement, highlighting the skin as a unique niche of incomplete immune reconstitution.

Donor-derived effector T cells seed the tissue-resident compartment after transplantation

Given the functional heterogeneity of TD_{RM}, we examined how subset composition evolves after transplantation. For further analysis, we first selected all T cell and NK cell clusters (Fig. 2a). T cells were categorized as CD4⁺ and CD8⁺ based on *CD4* and *CD8A/CD8B* expression levels, respectively, and annotated based on canonical genes (blood TS_B - *CCR7*, *SELL*; blood TD_B - *CCR7/SELL*¹⁰, *CD44*; skin TD_{RM} - *CD69*, *PRDM1*, *CREM*; blood and skin Treg – *Foxp3*, *CTLA4*) and following new nomenclature guidelines¹ (Fig. 2a,b). Additionally, we found a cluster representing a mix of unconventional T cells (T_{UC}) (Fig. 2a,b). 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 (Fig. 2b).

Integration of TCR- α/β sequencing data revealed a notable overlap with the annotated T cell clusters, detection across subsets and areas of clonal expansion within CD8⁺ TD_B and CD8⁺ TD_{RM} (Fig. 2c, Extended Data Fig. 2 a,b). In total we recovered 8,334 unique TCR- α/β clones. We next tested if clones are shared between sample groups (Extended Data Fig. 2 c). Only one clone was shared between patients and genotypes. However, between timepoints and tissues 143 and 145 clones were shared respectively, of which 31 matched both categories.

We detected few persistent clones in all patients following HSCT (Extended Data Fig. 2d). In accordance with the genotype analysis, persistent clones were preferably enriched in the skin without a detectable preference between CD4⁺ and CD8⁺ subsets (Extended Data Fig. 2d,e),

We extracted all T cells that shared clones in blood and skin from Baseline and post-HSCT (D14 and D100), 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 CD8⁺ TD_B and skin CD8⁺ TD_{RM}, indicating common ancestry of TD_{RM} and TD_B cells (Extended Data Fig. 2f). Next, we conducted a differential gene expression analysis comparing shared clones from blood and skin. We observed upregulation of several genes previously reported to be increased in human TD_{RM} compared to circulating T cells (*CD69*, *RUNX3*, *FABP5*, *PRDM1*, *LGALS3*, *CXCR4*, *VIM*)^{9,21-23}, whereas blood cells expressed genes of circulating effector cells (*SELL*, *CX3CR1*, *KLF2*)²¹ (Fig. 2d), indicating *de novo* TD_{RM} formation by donor T cells.

To assess whether most T cells in skin clusters represented bona fide TD_{RM} and upregulation of a resident phenotype occurred across all donor skin T cells, we computed a module score

for residency (TD_RM MS) based on canonical gene expression patterns that define TD_RM in humans and specifically in human skin^{21,23} (high expression: *CD69*, *PRDM1*, *EZR*, *LMNA*, *VIM*, *CXCR4*; low expression: *SELL*, *CCR7*, *CD27*, *TCF7*, *MAL*, *CX3CR1*, *S1PR1*). We then calculated the MS for all donor- and host-derived CD4⁺ and CD8⁺ TD_RM of the skin (Fig. 2e, Extended Data Table 1). Donor CD4⁺ TD_RM had comparable score values by D100, indicating residence, but displayed significantly lower values in GVHD, in line with effector or central memory T cell infiltration during inflammation. Among CD8⁺ TD_RM, the TD_RM MS was significantly reduced both at D100 and GVHD, suggesting a delayed return to a homeostatic T cell composition compared to CD4⁺ TD_RM and consistent with increased effector infiltration, which cluster with TD_RM upon inflammation (Fig. 2a,e).

In summary, individual donor-derived T cell clones can give rise to both TD_B and TD_RM progeny early after transplantation. On D100 and in GVHD donor cells in the skin partially acquire a resident phenotype, but display reduced TD_RM scores, likely reflecting a mixture of T cells transitioning to TD_RM and bona fide effectors.

Transplantation and GVHD drive a shift from T17- to T1-skewed TD_RM states

Given the emergence of new donor-derived TD_RM in the skin after transplantation, we next explored how TD_RM subset composition evolves over time. Differential gene expression analysis across all timepoints relative to Baseline revealed that GVHD was associated with the most pronounced transcriptional remodeling of skin T cells, while only modest changes were observed at Tx, D14 and D100 (Fig. 2f).

We focused on genes differentially expressed in skin TD_RM. The top 15 up- and downregulated genes per timepoint compared to Baseline revealed modest effects at Tx and D14, with upregulation of radiation response genes (*BAX*, *DDB2*, *APOBEC3H*)^{24,25}, while skin TD_RM were strongly perturbed in GVHD (Extended Data Fig. 2g,h). Exploring differentially expressed genes in detail, we found that many genes related to T cell residency, polarization and function were broadly dysregulated (Fig. 2g). Genes previously associated with TD_RM transcriptional or epigenetic signatures, such as *CCR4*, *LGALS3*^{9,23} in skin and *CREM* in lung²⁶ were downregulated, while genes linked to circulating effector T cells, like *SELL* and *KLF2*²¹ were upregulated in GVHD. These changes are consistent with the reduced resident module score (Fig. 2e) and increased infiltration of donor effector T cells during GVHD. Accordingly, GVHD-associated T cells displayed elevated expression of proliferation markers (*MKI67*, *AURKB*, *CDK1*) and type I and II interferon-response genes (*STAT1*, *BST2*, *JAK3*, *IFI6*) (Fig. 2g).

Strikingly, T17-associated markers, including *CCR6* and *IL7R*, were among the most strongly downregulated genes in both CD4⁺ and CD8⁺ TD_RM during GVHD (Fig. 2g), indicating a

selective impairment of T₁₇D_RM cells. In CD8⁺ T_DR_M, this was accompanied by reduced expression of murine T₁₇D_RM-master transcription factor *MAF*¹⁴ (encoding for c-MAF) and additional T17-related genes, including *AHR*²⁷ and *KLRB1*²⁸. Gene regulatory network analysis of GVHD samples further corroborated these findings (Fig. 2h), demonstrating diminished activity of T17-associated transcriptional programs (*RORC*, *BATF*, *AHR*, *STAT3*)^{27,29,30} alongside increased activation of canonical murine T₁D_RM-regulating networks (*EOMES*, *TBX21*, *ZNF683*)^{13,31}, particularly within CD8⁺ T_DR_M.

Collectively, these transcriptional profiles suggest that GVHD skin is characterized not only by a general shift from a tissue-resident, homeostatic signature toward an effector-dominated inflammatory phenotype, but also by a pronounced imbalance between T₁₇D_RM and T₁D_RM cells, particularly among CD8⁺ T_DR_M.

Resident skin cells favor the recruitment of type 1-polarized T cells in GVHD

To investigate signals contributing to the observed T₁₇D_RM/T₁D_RM imbalance, we analyzed differentially expressed genes in the skin microenvironment relative to Baseline. Among non-lymphoid compartments, Macrophages (MΦ), Dendritic cells (DC), and Fibroblasts (Fibro) displayed the most prominent transcriptional changes (Fig. 3a), so we explored differentially expressed genes and predicted cell-cell communication patterns between these cells.

GVHD lesions exhibited strong type I and II interferon response signatures (*STAT1*, *ISG15*, *IFI27*, *GBP1*, *GBP4*, *GBP5*, *SOCS1*, *IFITM1*), alongside downregulation of genes involved in tissue remodeling and extracellular matrix organization (*MMP1*, *MMP10*, *MMP19*, *ELN*, *SPARC*, *SELENOP*) (Fig. 3b-d). Notably, the CXCR3 ligands *CXCL9*, *CXCL10*, and *CXCL11* were among the most strongly induced genes and displayed increased communication probabilities, potentially suggesting predicted signaling activity, consistent with preferential recruitment of CXCR3-expressing T1-polarized cells during GVHD (Fig. 3b-d), in line with previous findings^{32,33}. Other ligand-receptor pairs with predicted interactions in GVHD included co-stimulatory and co-inhibitory signaling pathways (*CD80-CD28/CTLA4*, *LGALS9-CD44/HAVCR2*, *TNFSF9-TNFRSF9*, *NECTIN2/PVR-CD226/TIGIT*, *TNFSF4-TNFRSF4*) (Fig. 3e-g, Extended Data Fig. 3a-c), indicating a broadly activated and regulatory microenvironment.

Remarkably, *IL15* expression was already elevated in Fibro in uninflamed skin after HSCT and further upregulated in GVHD. In contrast, T_DR_M exhibited downregulation of *IL7R*, suggesting impaired responsiveness to IL-7 despite its local availability, whereas IL-15 signaling capacity may be preserved (Fig. 3e-g, Extended Data Fig. 3a-c). Consistent with murine studies demonstrating differential dependence of T₁₇D_RM and T₁D_RM on IL-7 and IL-15, respectively¹⁴,

these findings suggest that dysregulation of these signaling axes in the skin after HSCT may selectively impair T₁₇D_RM while favoring T₁D_RM. Overall, predicted communication toward CD8⁺ and CD4⁺ T_DR_M in GVHD was characterized by a relative reduction of *IL7R–IL2RG*, *CCR6*, and *CCR4*, and enhanced *CXCR3*- and *IL15RA–IL2RB*-mediated interactions (Fig. 3h, Extended Data Fig. 3d).

Immunofluorescence analysis confirmed these findings at the protein level (Fig 3i), revealing increased CXCL9/10 and IL-15 protein expression in CD45⁺ immune cells (with a similar trend in CD45⁻ cells (Fig. 3j-k, Supplementary Fig. S2a-b), and markedly reduced CD127 expression on CD3⁺ T cells (Fig. 3l, Supplementary Fig. S2c). Collectively, these data define a microenvironment that promotes recruitment and persistence of T1-polarized T cells while limiting signals required for T₁₇D_RM maintenance. These changes in cell-cell communication patterns can be associated with the T₁₇D_RM/T₁D_RM imbalance in the skin during GVHD, suggested by our single cell data.

T₁₇D_RM represent a distinct subset selectively affected by HSCT

Although T₁₇D_RM have been described in human skin, it remains unclear whether they employ the same developmental and maintenance mechanisms as T₁D_RM. Additionally, our differential expression analysis did not resolve whether gene expression shifts arise due to transcriptional reprogramming of cells or compositional shifts. The next goal was to better resolve CD8⁺ T₁₇D_RM and define their temporal and proportional dynamics following HSCT. To this end, we analyzed CD8⁺ T cell subsets (T_SB, T_DB and T_DR_M) and devised a T₁₇D_RM module score based on high expression of *IL7R*, *CCR6*, *RORC*, and *MAF*, and low expression of *CXCR3*, *IFNG*, *ZNF683*, and *TBX21*, reflecting T₁₇D_RM versus T₁D_RM conserved programs in mice¹⁴ (Fig. 4a,b). In line with previous analyses on healthy human skin³⁴, *IL17A* transcript recovery was low, and therefore not included in the module score. This score defined a group of CD8⁺ T₁₇D_RM, almost exclusively in the skin (Fig 4a,b).

Projection of CD8 T cells over time revealed a near-complete absence of cells in the T₁₇D_RM UMAP space during GVHD (Fig. 4c). Quantification of T₁₇D_RM frequency showed a reduction that did not reach statistical significance, likely due to limited statistical power of our small cohort (Fig. 4d). Differential expression analysis confirmed that T₁₇D_RM were enriched for canonical T17-associated genes (*STAT3*, *AHR*, *BATF*), as well as genes linked to lipid metabolism (*FAAH2*, *AGPAT4*, *PLCL1*) and resolution of inflammation and tissue repair (*ANXA1*, *NR3C1*, *ADAM19*) (Fig. 4e). In contrast, antiviral and cytotoxic genes (*CCL4*, *CCL5*, *XCL1*, *GNLY*, *NKG7*, *GZMA*, *GZMB*, *PRF1*) were reduced relative to other T_DR_M, which were correspondingly enriched for T₁D_RM genes, based on T17- MS and cytotoxic cells features (Fig. 4e).

Gene regulatory network analysis further supported activation of T17-defining transcription programs and reduced T1-associated networks in T₁₇D_{RM} (Fig. 4f), mirroring the global shift toward T1 polarization observed in GVHD (Fig. 2h). Together, our data identify human skin T₁₇D_{RM} as a transcriptionally and functionally distinct subset that is selectively depleted following HSCT and during GVHD.

As the module score did not uniquely define skin T₁₇D_{RM} in CD4⁺ T cells (Extended Data Fig. 4a-b), conclusions regarding CD4⁺ T_{DM} dynamics should be interpreted with caution. We show data regarding CD4 T cells for completeness and see similar trends (Extended Data Fig. 4c-f), but focus our primary conclusions on CD8⁺ T₁₇D_{RM}, where the module score showed a clearer discriminative capacity.

The disruption of T₁₇D_{RM} homeostasis may be mediated by IFN- γ -dominated microenvironment that antagonizes T17 polarization and the maintenance of T₁₇D_{RM}³⁵⁻³⁷. Moreover, the observed transcriptional changes in the skin microenvironment (Fig. 3b-d), including increased type I interferon signaling and upregulation of CXCL9/10 and IL-15 expression (Fig. 3b-d,j-l), may together favor recruitment and persistence of T1-polarized cells³⁵⁻³⁷.

While these findings define a T₁₇D_{RM}-like population and its dynamics at the transcriptional level, protein-level validation remained lacking. Therefore, we next sought to resolve skin T_{DM} subset heterogeneity and functional properties after transplantation at the protein level.

HSCT induces a GVHD-independent loss of CD8⁺ T₁₇D_{RM} in skin

To validate the reduction of T₁₇D_{RM} and assess functional changes at the protein level, we analyzed an independent HSCT cohort using flow cytometry (Fig. 5a). Given that the interval between D14 and D100 was not captured in our single-cell transcriptomics dataset and that GVHD typically arises within this window, we asked whether the reduction in T₁₇D_{RM} occurs as a general feature of post-transplant skin independent of GVHD.

We therefore included lesional GVHD biopsies, matched-timepoint GVHD-free HSCT samples and healthy donor controls (HD) (Fig. 5a). Using flow cytometry, we evaluated canonical T₁D_{RM}/T₁₇D_{RM} markers (CD69, CD103, CCR6, CD127, CD49a, ICOS) and key effector cytokines (TNF- α , GM-CSF, IL-17A, and IFN- γ) (Fig. 5b-h; Extended Data Fig. 5a-f).

At the single-marker level, CD8⁺ T cells displayed significant downregulation of CD69 after transplantation, consistent with a relative loss of bona fide T_{DM} and recent donor-derived T_D influx (Fig. 5b-c). CD127 was also markedly downregulated, while IFN- γ and CD49a were upregulated in both GVHD and GVHD-free samples. CCR6 was significantly decreased, with

a trend in GVHD-free skin (Fig. 5b-c). Of note, ICOS upregulation was specific to GVHD, whereas CD103, TNF- α , IL-17A, and GM-CSF remained largely unchanged (Fig. 5b-c), with overall low IL-17A expression, in line with previous reports^{15,17}.

Next, we performed unsupervised analysis to reveal coordinated changes in marker expression and to identify clusters affected by transplantation (Fig. 5 d-h). Phenograph clustering identified 10 clusters (Fig. 5e). Clusters 02, 06, and 09 exhibited T₁₇D_RM features (CD69+, CCR6+, CD127+), while clusters 01, 03, 07 and 08 resembled T₁D_RM (CD69, IFN- γ) with variable expression of CD103, CD49a, TNF- α , or GM-CSF (Fig. 5e,g,h). Cluster 10 showed mixed features with high CD103, suggestive of epidermal TD_RM (Fig. 5e,g,h). Differential abundance analysis revealed a significant enrichment of T₁₇D_RM clusters (02, 06) in HD compared to both GVHD and GVHD-free skin (Fig. 5f), while T₁D_RM-like clusters were enriched after transplantation (Fig. 5f). Notably, differences between GVHD and GVHD-free samples were modest (Fig. 5f). ICOS was broadly expressed across all clusters, not specific to T₁₇D_RM, as previously reported for murine skin¹⁴, suggesting a global increase in ICOS expression in GVHD and highlighting a potential divergence between human and murine TD_RM biology (Fig. 5g,h).

CD4⁺ T cells (excluding Tregs, Supplementary Fig. S3c) displayed similar individual marker changes, including reduced CD127 and CCR6 and increased ICOS and IFN- γ (Extended Data Fig. 5a), although selective depletion of T₁₇D_RM clusters was less pronounced.

Together, these data corroborate our transcriptomic analyses, demonstrating that T₁₇D_RM/T₁D_RM imbalance is a general feature of post-transplant skin, independent of clinically manifest GVHD, and may contribute to a pro-inflammatory niche that predisposes to or amplifies GVHD in susceptible individuals. Identification of T₁₇D_RM by surface markers independently of T1 marker expression (as opposed to the T17 module score) supports bona fide cellular depletion beyond transcriptional skewing.

Donor-derived T cells are impaired in reconstituting the CD8⁺ T₁₇D_RM compartment after transplantation

Given the replacement of host cells by donor cells in after HSCT, we next assessed chimerism dynamics within CD8⁺ TD_RM in our single cell transcriptomic dataset. Differential expression analysis revealed that donor TD_RM during GVHD exhibited increased cytotoxicity (*GZMA*, *GZMK*), activation (*HLA-DR*, *CD2*, *ICOS*), and co-inhibitory receptor expression (*TIGIT*, *CTLA4*, *HAVCR2*), while host TD_RM retained expression of genes associated with residency in mice (*ZNF683*, *BHLHE40*, *NR4A1*)^{13,38,39} (Fig. 6a,b).

In the HSCT setting without GVHD, fewer genes were differentially expressed, although donor TD_RM showed enrichment of cytotoxic markers (*GZMK*, *GZMH*), whereas host TD_RM preferentially expressed T17-associated genes (*CCR6*, *IL7R*, *BATF*)^{14,29,30} (Fig. 6a). Notably, donor cells were underrepresented within the T₁₇D_RM UMAP space (Fig. 6c, Fig. 4b) and failed to expand within this subset over time. While other donor TD_RM subsets increased by D100 and during GVHD, donor-derived T₁₇D_RM remained scarce (Fig. 6d). Conversely, host TD_RM persisted only at low levels overall, with T₁₇D_RM nearly absent (Fig. 6d).

Using CXCR3 and CD127 as surrogate markers, *in situ* analysis of matched Baseline and D100 samples showed a shift toward increased CXCR3+ and decreased CD127+ CD8+ T cells (Fig. 6e,f), consistent with T1 skewing, albeit not statistically significant. Combined immunofluorescence and fluorescence in-situ hybridization for sex-chromosomes (XY-FISH) in a sex-mismatched sample provided proof-of-principle evidence that host-derived CXCR3+CD8+ T cells can persist 100 days after transplantation (Fig. 6g,h),

Together, these findings indicate that donor-derived T cells are inefficient at generating or maintaining T₁₇D_RM, contributing to the observed subset imbalance after HSCT.

Circulating CD8+ T cells with ex-resident features mirror skin TD_RM remodeling

Since access to sex-mismatched HSCT skin samples was limited, we next determined whether TD_RM-derived recirculating ex-resident memory T cells (TD_{exR}) could serve as a surrogate for skin immunity. While CD4+ TD_{exR} (CD45RO+ or CD45RA-, CLA+, CD103+) have previously been shown to retain tissue-imprinted features after HSCT^{8,12}, CD8+ TD_{exR} have not been formally investigated in this context. We therefore analyzed this population as a potential surrogate for skin TD_RM. In healthy controls, CD8+ TD_{exR} closely mirrored skin TD_RM in expression of key markers dysregulated in GVHD, including CD127, CCR6, CD49a, and ICOS (Extended Data Fig. 6a-d).

In an independent HSCT cohort, blood-derived CD8+ TD_{exR} recapitulated post-transplant changes observed in skin, including significant downregulation of CD127 and upregulation of CD49a and ICOS (Extended Data Fig. 6e,f).

To assess chimerism, we sorted T17-like (CCR6+/CD127+) and T17^{lo} (CCR6-/CD127^{lo}) CD8+ TD_{exR} from sex-mismatched patients and performed XY-FISH (Extended Data Fig. 6g,h). In the GVHD and D100 samples, 7/8 patients had a higher fraction of CD8+ T₁₇^{lo}TD_{exR}, while T₁₇D_{exR} proportions were restored in long-term (>1 year) post-transplant patients (Extended Data Fig. 6i,j).

Although genotypic analysis was limited by low cell yields, most CD8⁺ TD_{exR} were donor-derived consistent with our single-cell data and in line with previously reported high heterogeneity in host chimerism, ranging from 0-100% in individual patients^{8,9}. While variability in chimerism precluded firm conclusions regarding subset-specific survival, these data support impaired acquisition of T17 features by donor cells. Overall, CD8⁺ TD_{exR} capture key phenotypic changes occurring in skin after HSCT and represent a promising peripheral biomarker for monitoring TD_RM dynamics.

Dysregulated CD8⁺ TD_RM phenotype normalizes subset imbalance resolves after long-term immune reconstitution

To assess long-term dynamics of skin TD_RM, we performed immunofluorescence analysis on skin sections from healthy controls, GVHD patients, and individuals 2–9 years post-transplantation (>1y pTx) (Fig. 7a). We focused on CD127 and ICOS as key dysregulated markers on CD8⁺ T cells in GVHD lesions (Fig. 7b-g). As expected, lesional GVHD skin showed reduced frequency and intensity of CD127 (Fig. 7b,c) and an increased ICOS expression (Fig. 7e,f). In contrast, patients in the >1y pTx group showed no evidence of persistent changes in these proteins, as levels were comparable to those seen in healthy skin (Fig. 7c,f).

Among these long-term post-transplant patients, prior GVHD history did not influence CD127 or ICOS expression (Fig. 7d,g), indicating that recovery of TD_RM homeostasis occurs irrespective of earlier disease, although statistical power was limited at these sample sizes.

Our data confirms that ICOS is not a reliable marker for T₁₇D_RM in human skin and cannot be used for their specific depletion in inflamed skin, as suggested in mice¹⁴, but rather marks inflamed skin lesions broadly. While CD127 is not exclusive to T₁₇D_RM, its dysregulation is a transient phenomenon. Although CD8⁺ CD127⁺ T cells (including T₁₇D_RM) are acutely depleted after transplantation, restoration of tissue homeostasis is associated with normalization of TD_RM subset composition, highlighting the plasticity of the skin TD_RM compartment.

DISCUSSION

TD_RM are critical in cancer, infection, vaccination, allergy and autoimmunity. Here, we dissect the developmental and functional heterogeneity of human skin TD_RM to inform strategies for their selective therapeutic modulation across clinical contexts.

Our data demonstrate a selective reduction of CD8⁺ T₁₇D_RM after HSCT, independent of GVHD, a T1-dominant microenvironment with upregulated CXCR3 ligands and IL-15

responses in lesional skin, impaired CD8⁺ T₁₇D_{RM} formation by donor cells and eventual recovery of T₁₇D_{RM}-associated markers long-term. The underlying mechanism could comprise myeloablative conditioning, leading to disrupted skin homeostasis⁴⁰⁻⁴², creating a pre-inflammatory, self-enforcing T1-skewed niche (CXCL9/10, IL-15, increased IFN-γ), concurrently suppressing the T17 lineage^{35,43,44}, and T₁₇D_{RM} generation and survival, even before GVHD-specific immune reactions begin. In this context, additional triggers such as minor tissue injury or alloantigen exposure could tip the balance toward GVHD in susceptible individuals. Notably, cell-cell communication and microenvironment analyses were only assessed in GVHD lesions, not matching GVHD-free samples, which were not part of the single cell cohort. Hence, the temporal order of these events, and whether T₁₇D_{RM} imbalance is a cause or consequence of the inflammatory microenvironment cannot be fully resolved from our cross-sectional comparisons.

Through clonal, genotypic and transcriptional analysis of host- and donor-derived CD8⁺ T₁₇D_{RM}, we observed delayed T₁₇D_{RM} generation post-HSCT and during GVHD, supporting a model where donor T₁₇D_{RM} readily form after transplantation but fail to replenish the T₁₇D_{RM} pool. While certain host T₁₇D_{RM} may persist in human skin for up to 10 years⁹, our study lacked statistical power to assess if host T₁₇D_{RM} are less stable than T₁D_{RM} post-transplant. Future studies in humans should address whether T₁₇D_{RM} have an inherently shorter lifespan than T₁D_{RM}. In mouse models, T₁₇D_{RM} decline fourfold within weeks after generation, while T₁D_{RM} remain stable¹⁴, attributed to higher Ki-67 and Bcl-2 expression in T₁D_{RM}¹⁴.

Murine T₁₇D_{RM} are crucial regulators of commensal microbiota and generated upon exposure to commensals^{15,16}. In humans, skin homeostasis involves constant microbial colonization. Potentially, T₁₇D_{RM} rely 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 conditioning⁴⁵. On the other hand, microbial dysbiosis, including *Staphylococcus* overgrowth in GVHD lesions⁴⁵ may result from the absence of T₁₇D_{RM}. This bidirectional regulation could be disrupted after HSCT and destabilize this circuit. Further research should clarify whether T₁₇D_{RM} contribute to GVHD pathogenesis or if it represents merely a consequence of transplantation.

The observed T₁₇D_{RM} depletion after HSCT may additionally be influenced by immunosuppressive drugs. Most patients received cyclosporine A (CSA) and mycophenolate mofetil (MMF), both of which target molecules involved in T17 differentiation, maintenance, and function⁴⁶⁻⁴⁸. MMF suppresses STAT3 phosphorylation *in vitro* and MMF-treated Lupus patients display lower levels of CCR6⁺ CD8⁺ memory T cells in blood⁴⁸. In mice, CSA limits the differentiation of pathogenic T17 cells⁴⁷. In addition, CSA inhibits activation-induced structural

changes in CD127, required for proliferation in response to IL-7, although only shown in bulk T cells *in vitro*⁴⁶. If applicable to skin T₁₇D_RM, this mechanism may further contribute to their post-transplant impairment.

Our study has limitations. Single-cell analyses involved relatively small cohorts and cell numbers, reflecting the difficulty of obtaining longitudinal skin biopsies from transplant recipients with limited size and low cell yield post-conditioning. We were unable to robustly assess host/donor chimerism among TD_RM subsets, and observed lower host chimerism than previously reported, where certain GVHD patients displayed >50% host skin T cells and host CD4⁺ TD_{exR}. This discrepancy likely reflects a small cohort size, considerable inter-patient variability and methodological differences and possibly results from different turnover kinetics of CD4⁺ and CD8⁺ host TD_RM in human skin^{8,9,12}.

Nevertheless, the use of rare, paired biopsies from the same patients before and after treatment, provides unique direct insight into human skin immune dynamics in a clinical setting. While we do not define the precise mechanisms underlying TD_RM pool changes, we validate these findings in additional cohorts, totaling samples from 85 HSCT patients, with in-depth discussion of potential functional implications.

Collectively, our study highlights the complexity and plasticity of skin TD_RM. The selective loss of CD8⁺ T₁₇D_RM after transplantation is intriguing, as it may contribute to GVHD and affects cell types central to inflammatory diseases like psoriasis¹⁷. While current cytokine-targeting therapies (TNF- α , IL-17 or IL-23 blockade) are effective, directly depleting the cytokine-producing cells could offer greater therapeutic potential. Additionally, methods to tailor TD_RM functions might enhance cell therapies, such as CAR T cells for solid tumors⁴⁹. Ultimately, these insights will be instrumental to design precision therapy approaches in inflammatory diseases and beyond.

MATERIALS AND METHODS

Human skin and blood samples

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, whereas GVHD-free samples were collected from HSCT patients without any symptoms of skin GVHD on the day of sampling at matched timepoints. Skin and blood samples from HSCT patients in the >1y pTx cohorts were taken at timepoints ranging from 1y – 9y after transplantation. Skin punch biopsies (6mm) were collected from healthy skin on the back or upper arms, or from lesional skin in the case of GVHD. Patients detailed clinical information is listed in Supplementary table S1. A detailed summary of which patient sample was used in which analysis is shown in Supplementary table S2. 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 FBS (Gibco, A5256801) with 10% 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 protocol⁵⁰. 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 Supplementary table S3), and the viability dye 7-AAD (Invitrogen, A1310) in FACS Buffer (DPBS, Gibco, 14190-169; 2 mM EDTA, Invitrogen,

10135423; 1% BSA, Sigma, A2153) 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% BSA 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 manufacturer's 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). 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 Supplementary table S2) was determined based on a custom adaptation of a Gaussian mixture model⁵¹. The full Cell Ranger filtered count matrices were imported and filtered by percentage mitochondrial reads ($\leq 10\%$), unique molecular identifiers (UMIs) per cell (≥ 800 counts) and detected genes per cell (≥ 600). Doublet cells were removed based on the HTO classification. The data were stored as Seurat (v.5.0.0) RDS or Scanpy⁵² (v.1.11.3) H5AD object for compatibility with R (v.4.4.1) and Python (v.3.10.16) respectively.

Data integration and dimensional reduction

Throughout the manuscript we employed scvi-tools⁵³ (v.1.2.2) single cell variational inference (scVI)⁵⁴ for data harmonization. All models were configured with a 30-dimensional latent space, two hidden layers with 128 nodes each, a negative binomial gene likelihood distribution and the sample ID as batch key. Further we informed the model with continuous covariates about biological (S-phase score, G2M-phase score) and technical variance (percentage mitochondrial reads). The latent space of the trained model was used to compute k-nearest neighbors (KNN) graph (30 neighbors), Leiden clustering⁵⁵ (resolution 1), and uniform manifold approximation and projection (UMAP) with Scanpy.

Cell type annotation

Cells passing the quality control thresholds were split into skin and blood subsets and annotated separately. The data were integrated and manually annotated using published marker gene lists. The annotation was further supported by CellTypist (v.1.1.0) automated cell type classification. Cluster with conflicting marker expression were further split for high resolution annotation. If the expression pattern strongly implied a cell type mixture, the respective cluster was removed from downstream analysis. The annotation yielded the final set of 84.447 high quality cells that were used for downstream tasks.

Annotation of T cells and NK cells is described in the Methods section *T cell nomenclature* and the main text. We additionally defined B cells (*MS4A1*), plasma cells (*CD79A*, *IRF4*) and *CD34*-expressing hematopoietic stem cells (HSC) (Supp. Figure 1). Among myeloid cells, monocyte clusters corresponded to classical (Mono_c: *CD14*), intermediate (Mono_{int}: *CD14*, *FCGR3A*) and non-classical monocytes (Mono_{nc}: *FCGR3A*) (Extended Data Fig. 1b,c), skin macrophages (MΦ) were defined by *CD68* and *F13A1* (Extended Data Fig. 1d,e). Dendritic cells (DC) segregated into classical DC subtypes (cDC1: *XCR1*, *CLEC9a*; cDC2: *CD1c*, *CLEC10A*; cDC_{mig}: *CCR7*, *LAMP3*), Langerhans cells (LC: *CD1A*, *CD207*) and plasmacytoid DC (pDC: *IL3RA*, *CLEC4C*) in the skin (Extended Data Fig. 1d,e), whereas in blood we only detected cDC2 and pDC (Extended Data Fig. 1b,c). Non-hematopoietic skin cells comprised 4 clusters of dermal fibroblasts (Fibro), which we annotated as F1 (superficial: *COL18A1*, *APCDD1*, *WIF1*), F2 (universal: *PI16*, *CD34*), F3 (FRC-like: *IL33*, *CCL19*) and F4 (hair-follicle associated: *TNN*, *COCH*, *CRABP1*) in line with a recently proposed consensus annotation for human skin fibroblasts⁵⁶ (Extended Data Fig. 1d,f). We also detected vascular and lymphatic endothelial cells (vEC: *PECAM1*, *CD74*; IEC: *LYVE1*, *CCL21*), alongside two pericyte clusters (PC1: *RERGL*, *ACTA2*, PC2: *ACTA2*, *CCL26*) (Extended Data Fig. 1d,f). Epithelial cells comprised basal keratinocytes (KC_b: *KRT14*, *KRT15*) and suprabasal keratinocytes (KC_{sb}: *KRT1*, *KRT10*) as well as melanocytes (Mlc: *MLANA*) (Extended Data Fig. 1d,e). We then used this high-resolution cluster information to recalculate the uniform manifold approximation and projection (UMAP) and to condense the subclusters into broad immune and non-hematopoietic cell type classes, characterized by canonical marker expression (Fig. 1e,f), thereby generating two annotation levels to facilitate both broad comparisons and subpopulation-specific analyses.

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-lite⁵⁷ (v.1.2.3) 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. For cell genotype demultiplexing we utilized vireoSNP²⁰ (v.0.5.8) 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. To determine variants with the highest predictive power for genotype demultiplexing we utilized GTbarcode from vireoSNP.

TCR analysis

Cell Ranger filtered contig annotation files were imported using Scirpy (v0.22.1) and restricted to cells containing exactly one paired $\alpha\beta$ TCR. The adaptive immune receptor repertoire (AIRR) data were converted into TCRdist-compatible format (v0.2.2). Following deduplication, a TCRrep object was initialized, and clonotypes were defined based on exact matching of α - and β -chain V genes, J genes, and CDR3 sequences. To identify shared (“public”) TCRs, a graph was constructed from the TCRdist distance matrix and pruned to retain edges corresponding to distances ≤ 36 TCRdist units. Graph-based clonotypes (“meta-clones”) were defined as connected components within this radius. The respective clone identifiers were subsequently mapped back to the single-cell level for downstream analysis. This more permissive clonal definition modestly increased the sharing of meta-clones across patients and longitudinal timepoints, while no increase in sharing was observed between tissues (data not shown). Therefore, subsequent analysis was restricted to exact clonotypes to preserve specificity.

Differential expression analysis (DEA)

For DEA we employ the edgeR⁵⁸ (v.3.36.0) function voomLmFit which adapts the Limma⁵⁹ (v.3.50.3) workflow to moderate abundant zero counts in single cell data with pseudobulks^{60,61}. Briefly, the count matrix was split by cell type, and samples with less than three cells were removed. Next, the expression matrix was filtered to only retain genes with three counts in at least three samples. At this step we also dropped covariates with less than three samples. For each cell type, a model was fitted with patient ID included as a blocking variable. 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-regulatory network analysis (GRN)

For the GRN analysis we used pyscenic^{62,63} (v.0.12.1) with the GRNBoos2⁶⁴ algorithm and followed the Nature Protocol⁶³. The analysis was performed only on T cell clusters.

Cell-cell interaction (CCI) analysis

For CCI we used CellChat (v.2.1.2)⁶⁵ with the human database subset to Cell-Cell Contact and Secreted Signaling pathways. CellChat objects were constructed per timepoint and the full feature space without overexpression analysis to preserve sensitivity for rare cell types with limited cell numbers. For the communication probability the expression was trimmed by the truncated mean and threshold at 0.01. Significant ligand-receptor (LR) interactions were determined ($p\text{-value} \leq 0.05$) for downstream analysis. To validate regulatory perturbation, we combine the LR interactions with differential expression results ($p\text{-value} \leq 0.05$).

Differential cell type abundance testing

We constructed the miloR (v.2.2.0) graph using the scVI latent space derived from skin and blood cells. A neighborhood parameter of $k=30$ was selected, resulting in an average neighborhood size of approximately 50 cells. Each timepoint was compared to Baseline and tested for patient-specific enrichment. False discovery rate (FDR) correction was performed using graph-overlap weighting.

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% FBS (Gibco, A5256801), 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. Stained samples were acquired on a CYTEK Aurora Spectral flow cytometer (5 Laser) at the Flow Cytometry Core Facility of MedUni Vienna. Antibodies used in these experiments are listed in Supplementary table S3. The exact antibody panels and gating strategy are shown in Supplementary table S5 and Supplementary Figure S3. Data was analyzed using FlowJo (v10.9.0) or OMIQ (Dotmatics) software. Conventional gating was done in FlowJo, using fluorescence-minus-one samples and unstimulated samples as gating controls. Samples with <50 cells in parent population (CD8⁺ T cells or CD4⁺ T cells) were excluded from downstream gating, marked in orange in respective plots. Calculations of opt-

SNE embedding and Phenograph clusters were run in OMIQ with software-integrated packages. For this purpose, down-sampling of maximum 10,000 cells per group was applied before embedding. Diffcyt package with EdgeR in OMIQ was used to determine cluster enrichment.

Immunofluorescence profiling of human skin sections

For *in situ* staining of skin cells, we prepared skin sections of OCT-embedded fresh-frozen tissue. Sample blocks were cut to 6-8 μm sections on adhesion slides (Epredia, J1800AMNZ) on a Leica Cryostat and acetone-fixed (10 min on wet-ice) for cryo-preservation. For staining, slides were blocked with 2% goat serum (Dako, X0907) for 30 min, either with or without 0.3% Tween 20 (Biorad, 1706531) for permeabilization, depending on targets. Slides were stained with primary unlabeled or fluorescently labelled antibodies, or isotype-matched control antibodies for 2-3h at room temperature or overnight at 4°C and subsequently stained with appropriate secondary antibodies for 30 min at room temperature in 1X DPBS (PanReac AppliChem, A0964,9100) with 2% BSA (Sigma, A2153). Antibodies used in these experiments are listed in Supplementary table S3, exact panels for each analysis are listed in Supplementary table S4. Slides were counterstained for 5 min with DAPI (Sigma, D9542-1MG) in DAPI with 2% BSA 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 20X 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 2 sections or a minimum area of 1 mm^2 was analyzed.

Cell sorting for cytopins

For sorting TD_{exR} subsets, frozen PBMC were thawed and rested overnight in RPMI1640 Medium (Gibco, 52400-025) with 1% Penicillin/Streptomycin (Gibco, 15140122) and 10% FBS (Gibco, A5256801) in 6-well plates at 37°C, 5% CO_2 . On the next day, cells were washed in cold DPBS and stained for fluorescence activated cell sorting, as described above. $\text{T}_{17}\text{D}_{\text{exR}}$ and $\text{T}_{17\text{lo}}\text{D}_{\text{exR}}$ were sorted at a BD FACS Aria III or a Beckman Coulter Cytoflex SRT into 100 μl FACS Buffer. The exact antibody panels and gating strategy are listed in Supplementary table S5 and Supplementary Figure S3, respectively. We then performed cytopins of sorted cells on adhesion slides (Epredia, J1800AMNZ). Cytofunnels (Epredia, 5991040) were equilibrated by spinning 100 μl DPBS for 5 min at 600 rpm in a Shandon Cytospin 3 centrifuge. Sorted cell suspensions were added to cytofunnels and spun for 2 min at 600 rpm. Slides were air-dried for 10 min and frozen at -20°C.

X/Y-chromosome fluorescence in situ hybridization (XY-FISH)

For XY-FISH, we used the CytoCell FAST FISH Prenatal X, Y and 18 Enumeration Probe Kit (Oxford Gene Technology IP Limited, LPF002). FISH on both sorted cytopsin samples and skin sections was performed according to the manufacturer's protocol with the following modifications: For pretreatment, slides were thawed for 10 min in DPBS (PanReac AppliChem, A0964,9100) at room temperature, and then digested in pepsin working solution for 13 min at 37°C. Ethanol washes were performed for 2 min, and prior to probe spotting, slides were incubated in 70% Formamide (Honeywell, Riedel-de Haen, 47670) in 1X SSC Buffer (Sigma, S6639) for 2 min at 75°C, followed by dehydration in an ethanol series. The sample area was circled with a heat-resistant hydrophobic barrier pen (Sigma, Z377821) prior to spotting 1.5-2 µl of pre-warmed probe onto pre-warmed slides, the cover slip was sealed with Leukofix tape (Leukoplast, 0212200). Slides were then denatured at 81°C in a Thermocycler for 5 min and incubated in a humid, light protected chamber at 37°C, 5% CO₂ overnight. The next day after post-hybridization washes, cytopsin slides were mounted with 5 µl DAPI antifade (Oxford Gene Technology IP Limited, LPF002), whereas skin slides were stained for 5 min with DAPI (Sigma, D9542-1MG) in DPBS and mounted with PermaFluor Eprelia Lab Vision aqueous mounting medium (Eprelia, TA-030-FM). Slides were stored at 4°C and acquired within two days on a TissueFAX inverted fluorescent slide scanner microscope (TissueGnostics) with the 20X objective and analyzed using StrataQuest 8 software, using software-integrated dot detection (TissueGnostics). For quantification, only cells with detection of either one red and one green dot (male) or two green dots (female) were included. Samples with a detection limit of more than 5% host chimerism due to low overall cell counts were excluded. FISH on previously IF-stained skin sections was performed using the same protocol, with an additional 10-min incubation step in 1X SSC buffer prior to the first DPBS wash to allow cover slip removal.

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. All boxplots show the mean and standard error of mean (SEM).

T cell nomenclature

We applied a modular nomenclature for T cell subsets based on recent consensus guidelines¹. This system uses a combination of abbreviations and subscripts to denote migration capacity, tissue location, memory state, and functional properties. In this study, migration and tissue location are indicated by: S (secondary lymphoid organ, characterized by *CCR7*, *SELL*), D (disseminated, *CCR7*^{lo} *SELL*^{lo}), with subscripts _B (isolated from blood), and _R (resident, isolated

from skin, expressing *CD69*, *ITGAE*, *PRDM1*, *CREM*). Memory properties are indicated by adding M. Functional properties are denoted by superscripts: _{reg} (regulatory), ₁₇ (type 17 polarized), and ₁ (type 1 polarized). For single-cell RNA sequencing, T cells were annotated according to this system based on their transcriptional profiles (Fig. 2a,b). Additionally, a cluster of unconventional T cells (T_{UC}) expressed *CD3D* but lacked *CD4* and had low *CD8A* and *CD8B*, but expression of genes such as *ZBTB16*, *FCGR3A* and genes coding for $\gamma\delta$ T cell receptor, indicative of a mixed population of MAIT, NKT and $\gamma\delta$ T cells. All other subsets were distinguished as CD4+ or CD8+, based on transcripts or protein level expression.

For flow cytometry analyses, TD_{RM} were identified by expression of CD69 and/or CD103, with subsets $T_{17}D_{RM}$ and T_1D_{RM} defined as CCR6+CD127+ and IFN- γ + and/or CD49a+, respectively. Recirculating T cells with resident phenotype (previously also termed cTRM in humans⁸ or ex-TRM in mice⁶⁶) were designated TD_{exR} , with the same functional subscript definition as TD_{RM} . Detailed gating strategies for flow cytometry are provided in Supplementary Figure S3.

Figures

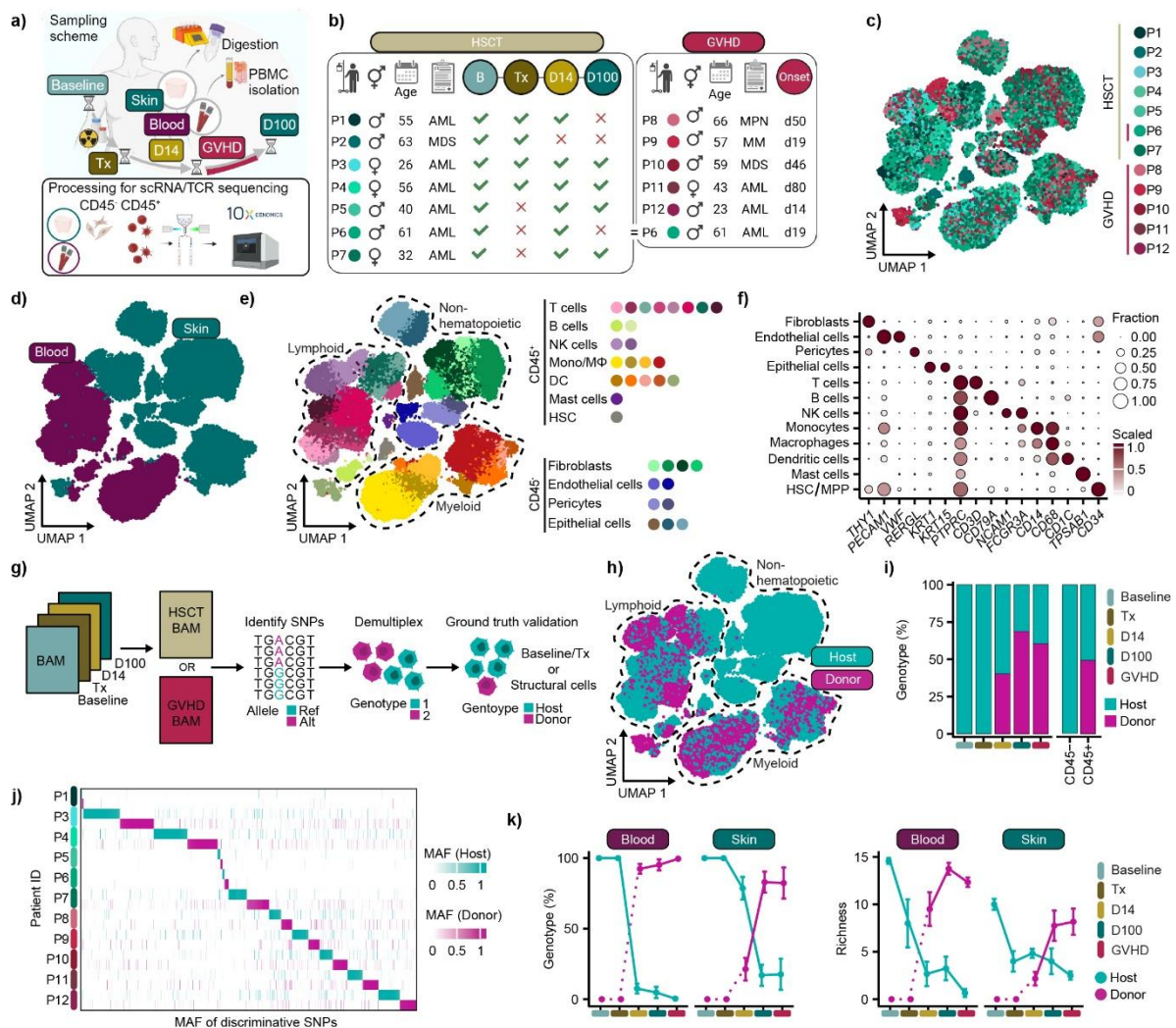


Figure 1 –Single-cell RNA sequencing analysis of a time-, tissue-, cell type- and genotype-resolved dataset during HSCT and GVHD

a) Sampling and sorting strategy for single-cell transcriptomic profiling. b) Patient sample overview. c-e) UMAP of all cells recovered from single-cell RNA sequencing, colored by (c) Patient ID, (d) tissue source and (e) cell type clusters. f) Dot plot of marker gene expression for broad cell type annotation. g) Strategy for SNP-based host/donor genotype demultiplexing. h) UMAP colored by host/donor genotype. i) Host/donor cell percentage across timepoints and CD45⁺/CD45⁻ cells. j) Minor allele frequency of discriminative host/donor SNPs per patient. k) Host/donor percentage (left) and cell type richness (right) among CD45⁺ immune cells over timepoints (mean + SEM).



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CD215+ (k) and CD127+ (l) cells among CD3+ T cells and CXCL9/10+ (j), IL-15+ (k) and IL-7+ (l) among CD45+ and CD45- skin cells in the upper dermis (Unpaired t-test; p-val: * ≤ 0.05 , *** ≤ 0.001 , ns = not significant; dots represent individual samples, mean $\pm$ SEM).

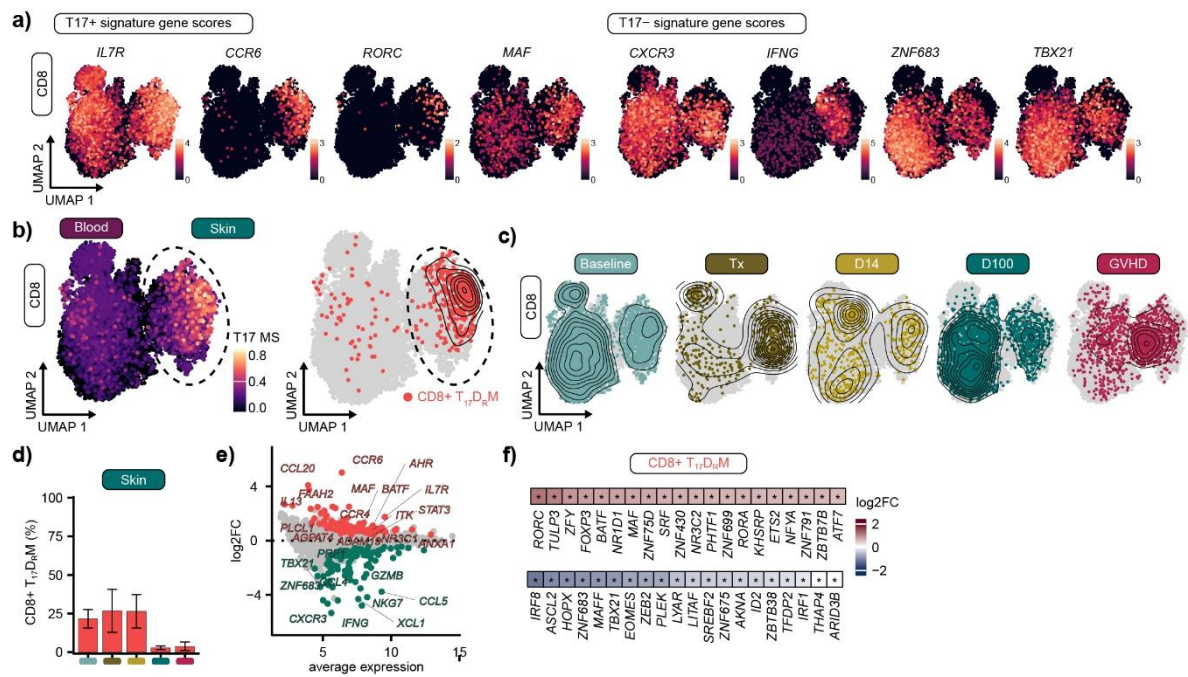


Figure 4 – CD8+ T cell transcriptional analysis to investigate changes in T₁₇DRM

a) UMAP of CD8+ T cells after re-integration showing expression of T17module score (MS) component genes. b) UMAP of T17 MS values (left) and classification of T₁₇DRM (right) among CD8+ T cells. c) Density maps of CD8+ T cells across sampling timepoints. d) CD8+ T₁₇DRM frequency over timepoints (ANOVA, not significant, mean $\pm$ SEM). e) Volcano plot of DEG between T₁₇DRM (orange) vs remaining TDRM (green) (adjusted p-val ≤ 0.05). f) Top 20 regulons differentially active in CD8+ T₁₇DRM vs remaining TDRM (adjusted p-val ≤ 0.05).

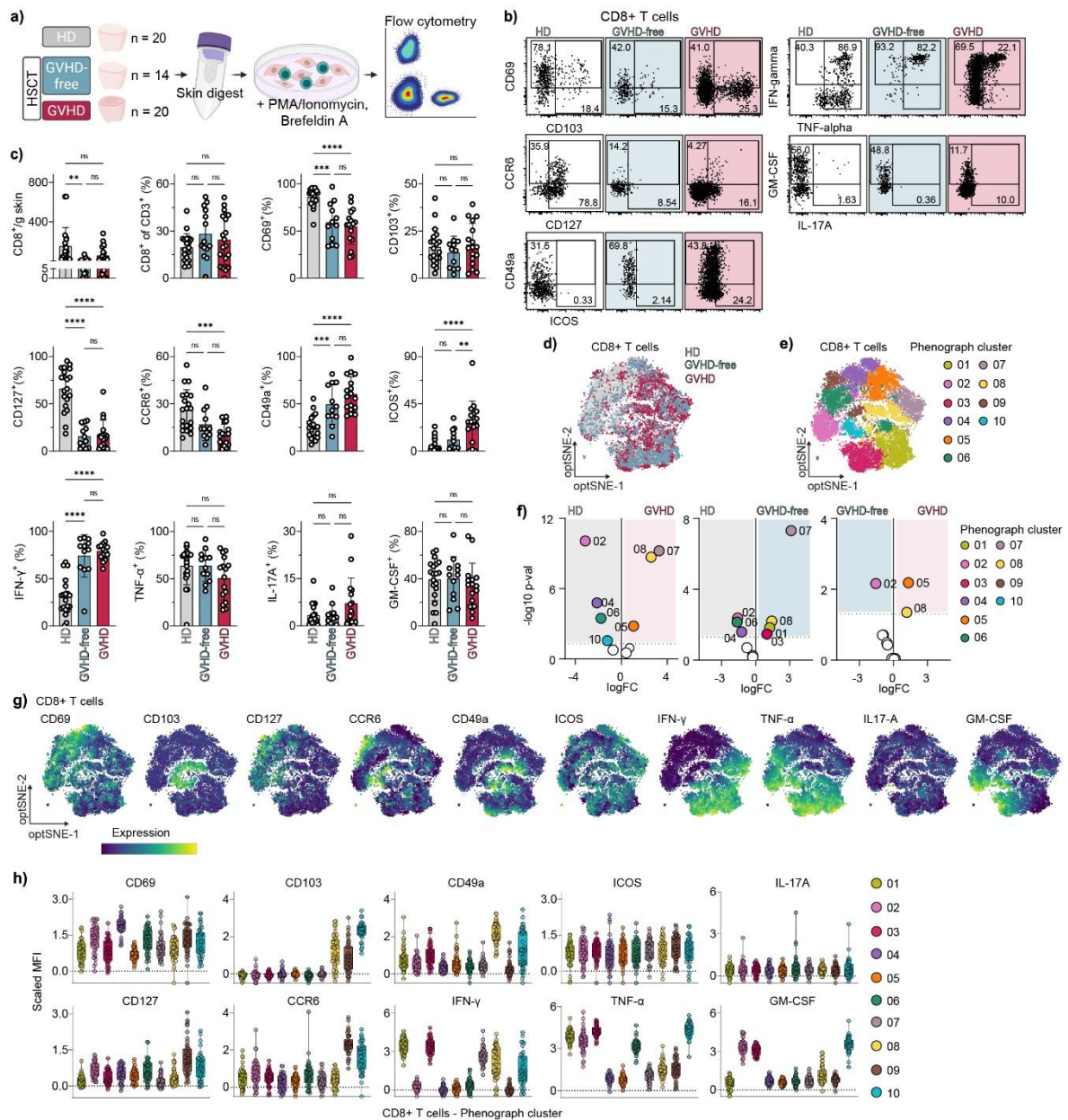


Figure 5 – Phenotypic and functional profiling of skin CD8 $^{+}$ T cells after transplantation

a) Schematic for T cell profiling in skin biopsies of healthy donors (HD), GVHD-free HSCT patients and GVHD. b) Representative gating strategy surface markers and cytokines. c) Quantification of CD8 $^{+}$ T cell markers and cytokines across groups (One-way ANOVA with Tukey's multiple comparisons test; p-val: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 , ns = not significant; dots represent individual samples, n=14-20, mean $\pm$ SEM). d-e) Opt-SNE projection of skin CD8 $^{+}$ T cells (n=17,194) colored by (d) sample group or (e) Phenograph cluster. f) Volcano plot of differentially abundant clusters between indicated comparisons (adjusted p-val ≤ 0.05). g) Opt-SNE feature plots of scaled mean fluorescence intensity per parameter. h) Boxplots showing scaled mean fluorescence intensity per parameter across Phenograph clusters.

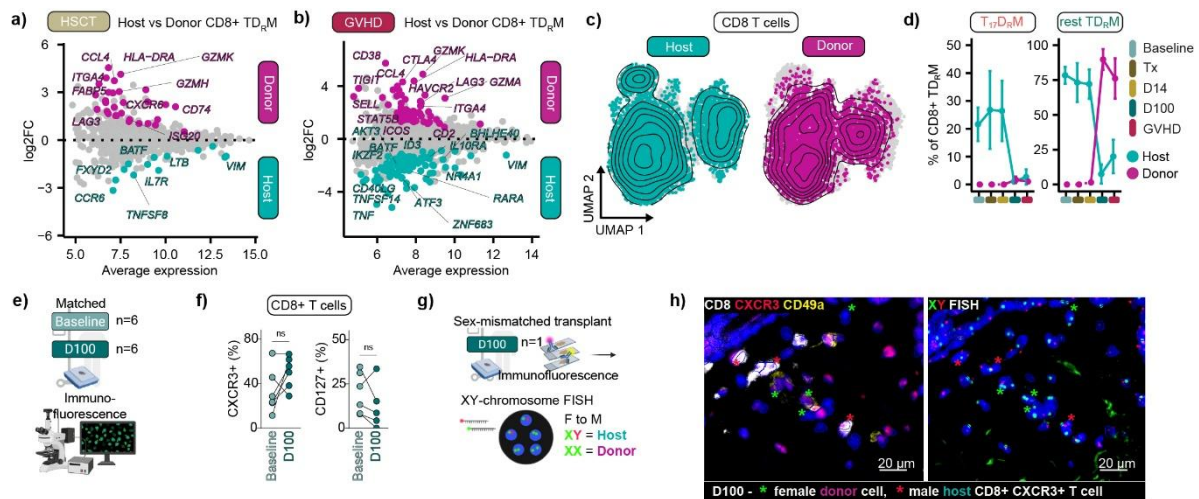


Figure 6 – Determining differences in host and donor CD8+ TDRM after transplantation

a-b) Volcano plots of DEG between host and donor CD8+ TDRM at (a) HSCT (D14+D100) and (b) GVHD (adjusted p-val ≤ 0.05). c) Density maps of host and donor CD8+ T cells. d) Percentages of host and donor cell CD8+ T17DRM and remaining TDRM over timepoints (mean $\pm$ SEM). e) Schematic of IF on matched HSCT skin samples. f) Percentage of CXCR3+ and CD127+ cells among CD8+ skin T cells (Paired t-test, not significant). g) Schematic of sequential IF and XY-FISH on a D100 skin sample from a sex-mismatched transplant recipient. h) Representative image of skin cells stained for CXCR3, CD49a and CD8 with XY-chromosome FISH, showing mixed chimerism in same tissue region.

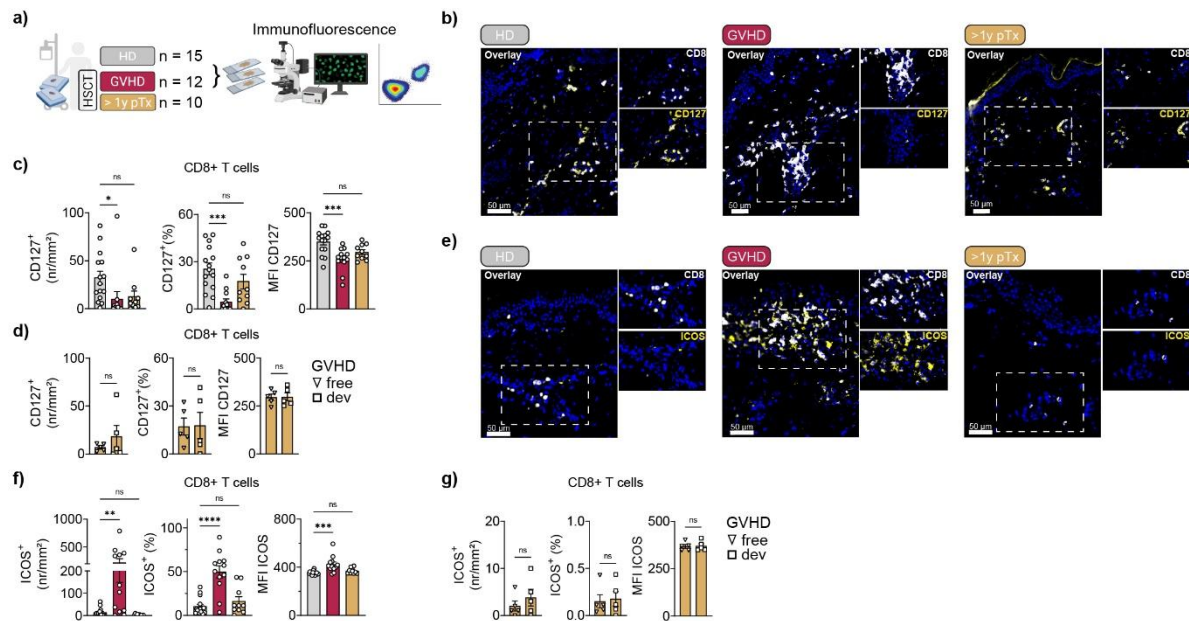
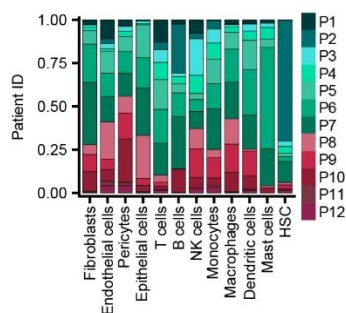


Figure 7 – Assessment of skin T cell phenotype recovery after HSCT

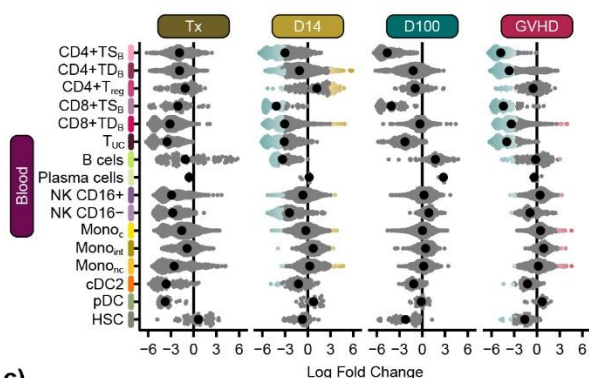
a) Schematic of IF on skin biopsies from healthy donors (HD), patients 2-9 years after transplantation (>1y pTx) and lesional skin of GVHD patients (GVHD). b) Representative images of CD8 and CD127 staining. c) Quantification of CD8⁺ CD127⁺ cell density (left), CD127⁺ percentage among CD8⁺ cells (middle) and CD127 MFI on CD8⁺ cells (right). d) Same analysis as (c), comparing GVHD-free patients (n=5) and prior GVHD developers (n = 5) within <1y pTx group. e) Representative images of CD8 and ICOS staining. f) Quantification of CD8⁺ ICOS⁺ cell density (left), ICOS⁺ percentage among CD8⁺ cells (middle) and ICOS MFI on CD8⁺ cells (right). g) Same analysis as in (f), comparing GVHD-free patients (n=5) and prior GVHD developers (n = 5) within <1y pTx samples. Statistical tests: (c,f) One-way ANOVA with Dunnett's multiple comparisons test (HSCT groups vs. HD); (d,g) Unpaired t-test between GVHD-free and GVHD developers. Dots represent individual patients (p-val: * ≤0.05, ** ≤0.01; *** ≤0.001, **** ≤0.0001, ns = not significant, mean ± SEM).

Extended Data

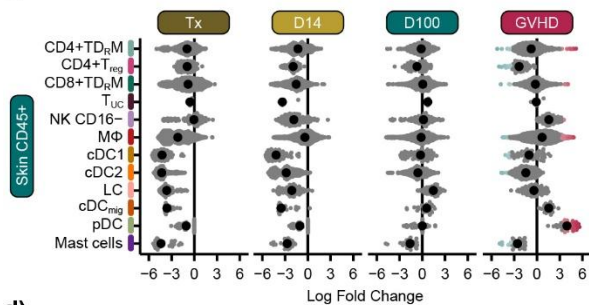
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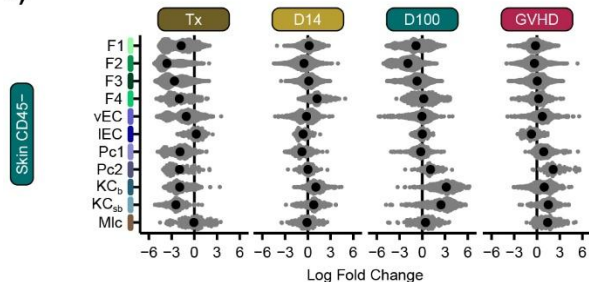
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c)

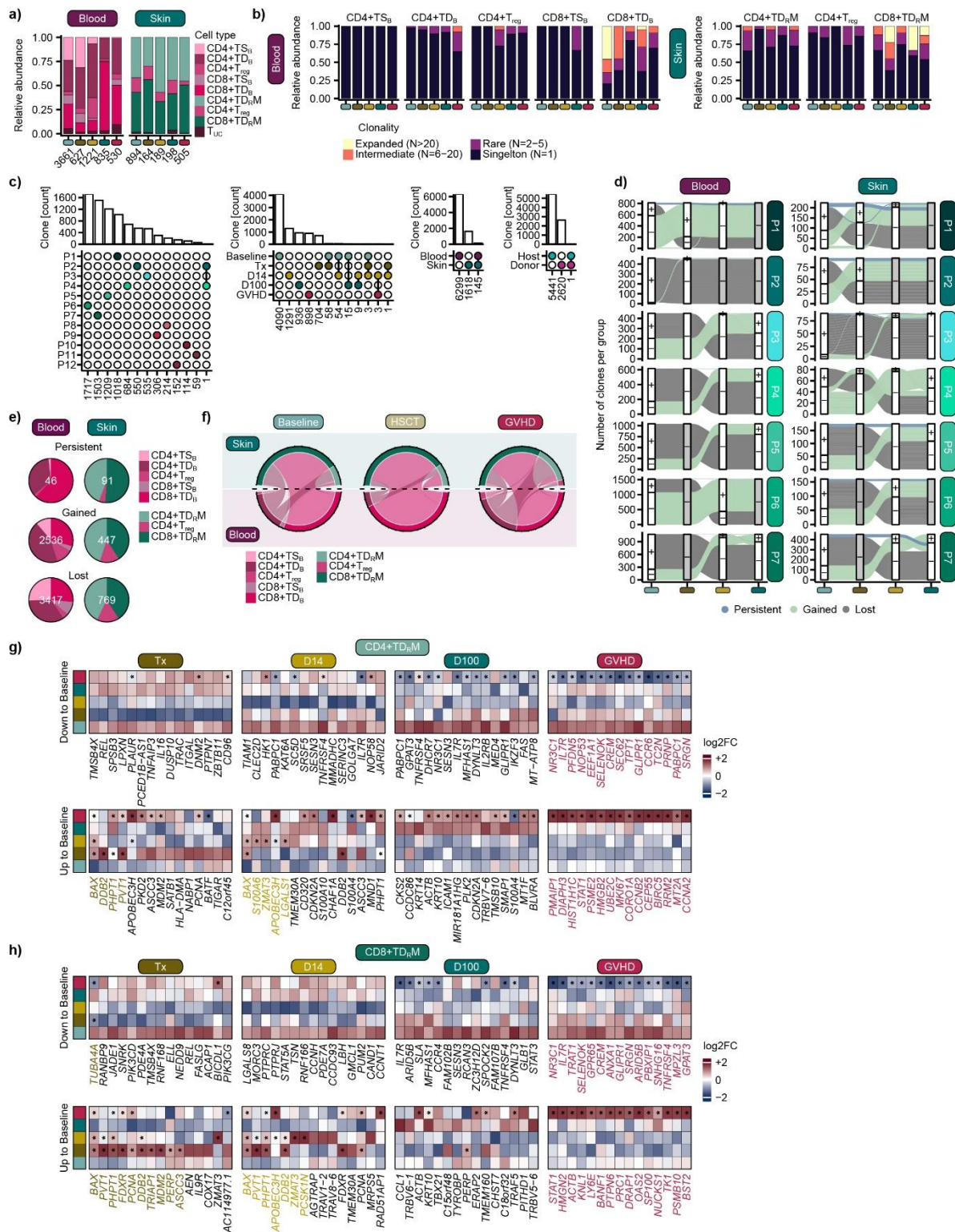


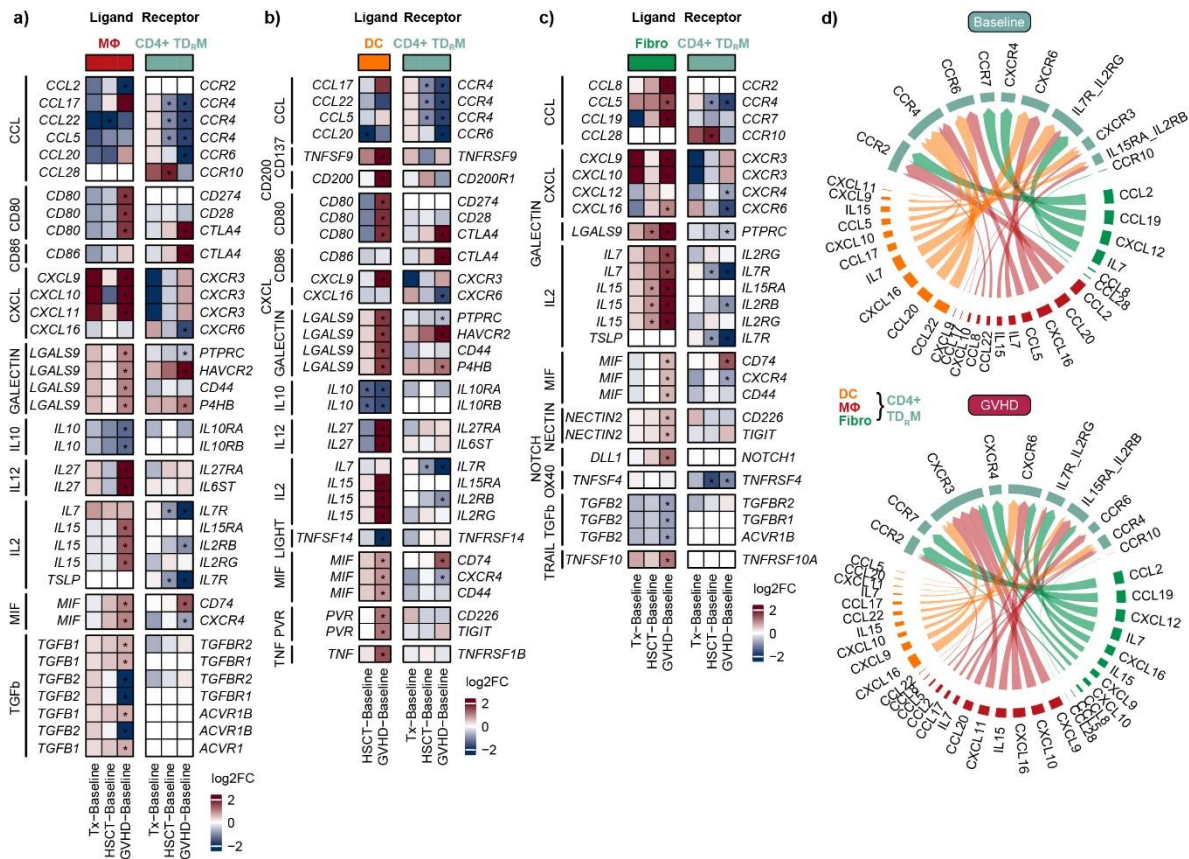
d)



Extended Data Figure 1 – Cell recovery and compositional shifts in identified subsets

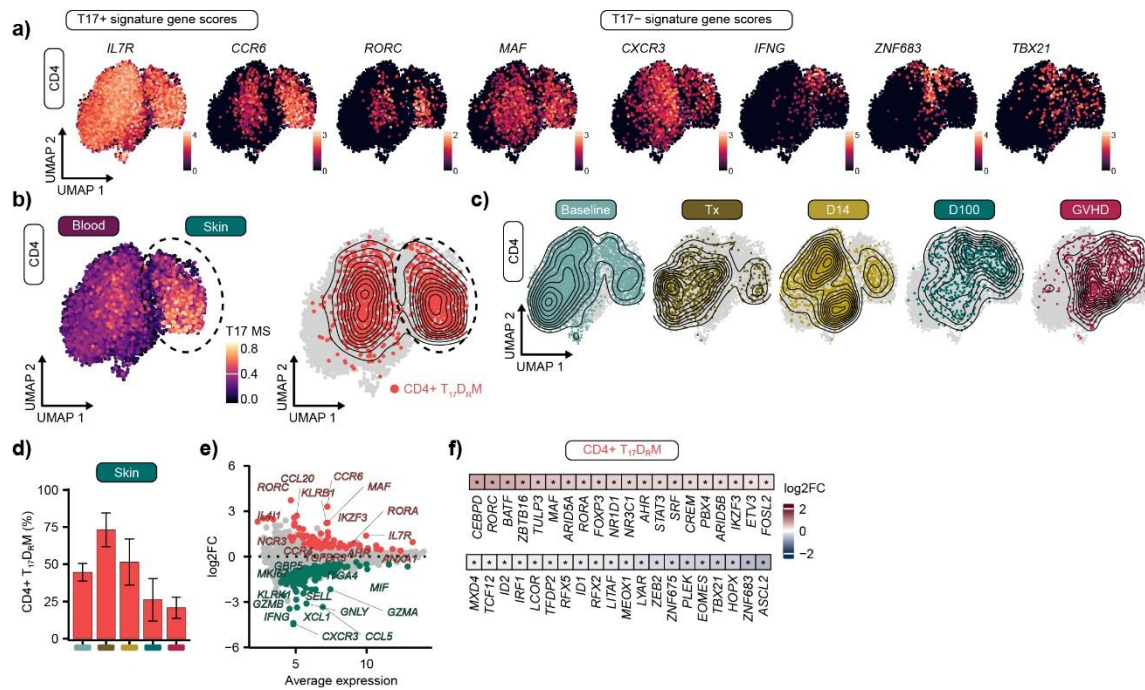
a) Bar chart of relative frequency of patient sample representation in each cell group in scRNA-seq data. b-d) Log fold changes of differential cell type abundance testing per timepoint across annotated cell types within (b) blood, (c) skin CD45+ cells and (d) skin CD45- cells. Colored dots indicate neighborhoods significantly enriched in one group ($p\text{-val} \leq 0.05$). Black dots represent the mean abundance shift across neighborhoods





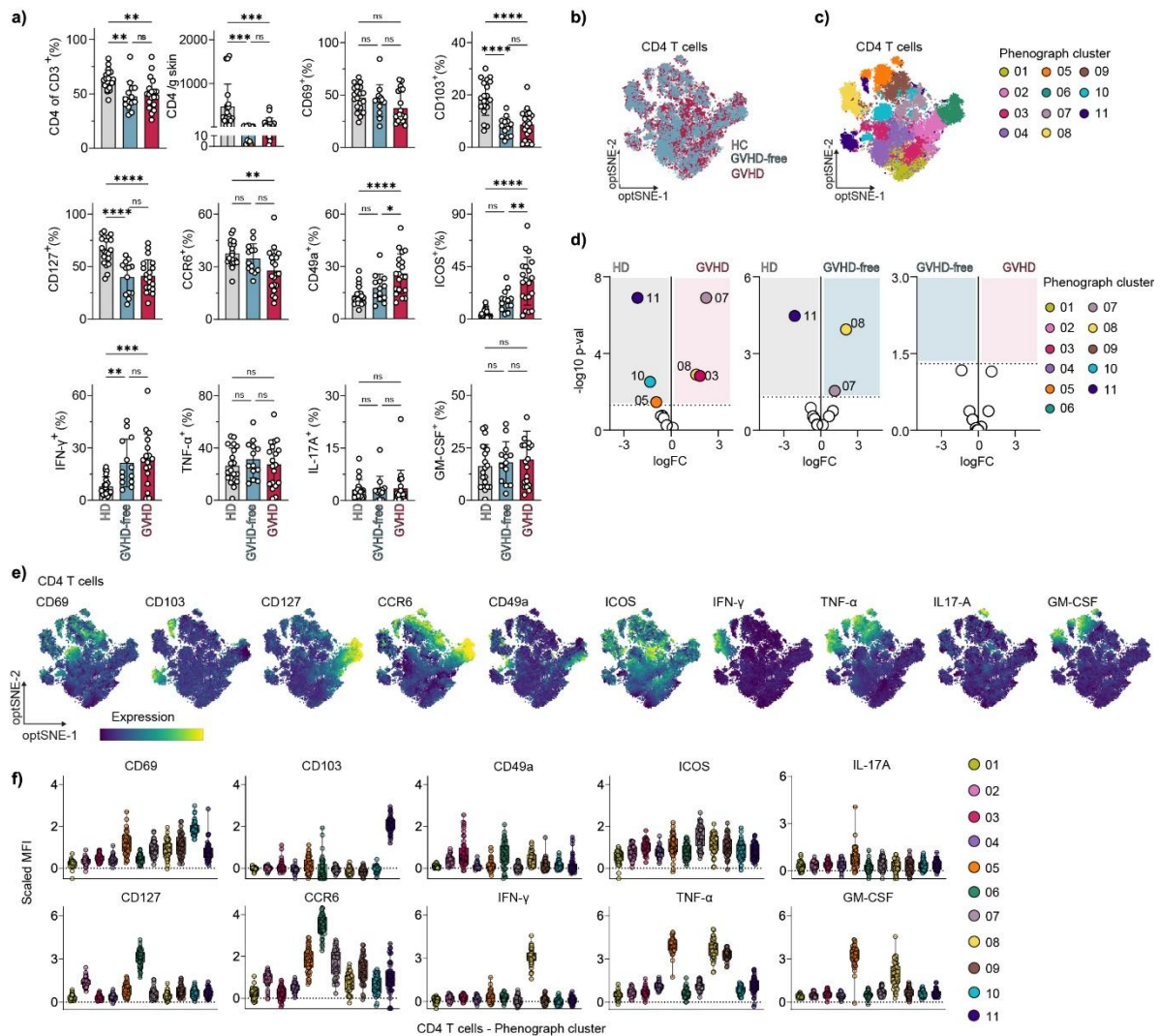
Extended Data Figure 3 – Cell-cell communication of Macrophages, DC and Fibroblasts with CD4+ TDRM

a-c) Ligand-receptor interaction maps from a) MΦ, b) DC and c) Fibro to CD4+ TDRM showing differential regulation vs. Baseline ($p\text{-val} \leq 0.05$). d) Chord diagrams of relative CCL, CXCL and IL2 signaling strength from MΦ, DC and Fibro to CD4+ TDRM at Baseline (top) or GVHD (bottom).



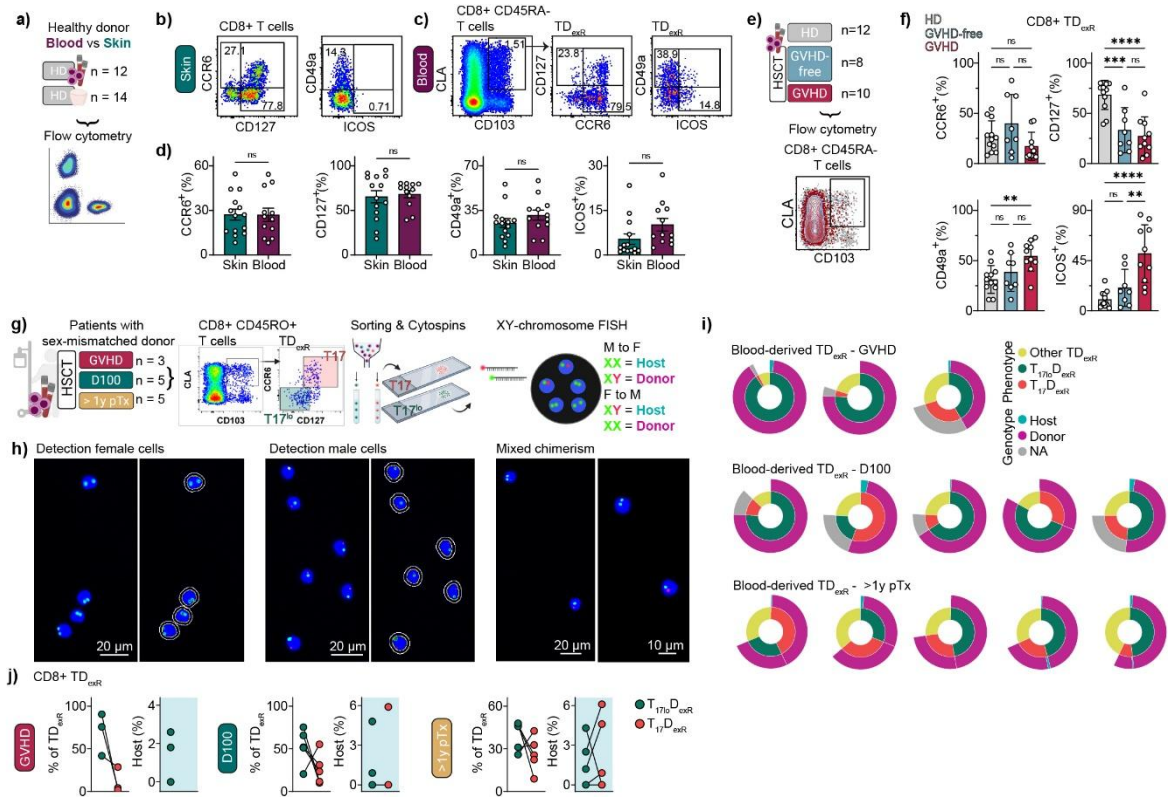
Extended Data Figure 4 – CD4+ T cell transcriptional analysis to investigate changes in T17DRM

a) UMAP of CD4+ T cells after re-integration showing expression T17 module score (MS) component genes. b) UMAP of T17 MS values (left) and CD4+ T17DRM classification (right). c) Density maps of CD4+ T cells across sampling timepoints. d) CD4+ T17DRM frequency over timepoints (One-way ANOVA, not significant, mean $\pm$ SEM). e) Volcano plot of DEG between CD4+ T17DRM and remaining CD4+ TDRM (adjusted p-val $\leq$ 0.05). f) Top 20 regulons differentially active in CD4+ T17DRM vs remaining CD4+ TDRM (adjusted p-val $\leq$ 0.05).



Extended Data Figure 5 – Phenotypic and functional profiling of skin CD4 T cells after transplantation

a) Quantification of CD4 T cell markers and cytokines in HD, GVHD-free and GVHD groups (One-way ANOVA with Tukey's multiple comparisons test between all groups; p-val: * ≤ 0.05 , *** ≤ 0.001 , ns = not significant; dots represent individual samples, n=14-20, mean $\pm$ SEM). b-c) Opt-SNE projections of skin CD4 T cells (n=22,986) colored by (b) sample group or (c) Phenograph cluster. d) Volcano plot of differentially abundant clusters between indicated comparisons (adjusted p-val ≤ 0.05). e) Opt-SNE feature plots of scaled mean fluorescence intensity per parameter. f) Boxplots showing scaled mean fluorescence intensity per parameter across Phenograph clusters



Extended Data Figure 6 – Analysis of phenotype and genotype in CD8+ TD_{exR}

a) Schematic for comparing skin T cells and blood TD_{exR} in healthy donors. b) Gating strategy for T cell markers on skin CD8+ T cells. c) Gating strategy for TD_{exR} among blood memory CD8+ T cells and markers expression. d) Percentage of CD127⁺, CCR6⁺, CD49a⁺ and ICOS⁺ cells among skin CD8+ T cells vs. blood CD8+ TD_{exR} (Unpaired t-test, not significant, mean ± SEM). e) Schematic for comparing TD_{exR} marker expression across HD, GVHD-free and GVHD groups. f) Percentage of CD127⁺, CCR6⁺, CD49a⁺ or ICOS⁺ cells among CD8+ TD_{exR} per groups (One-way ANOVA with Tukey's multiple comparisons test, p-val: ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001, ns = not significant, mean ± SEM). g) Schematic for sorting CD8+ T17 and T17^{lo} TD_{exR} from sex-mismatched HSCT patients, followed by cytopsin and XY-FISH. h) Representative images of FISH spot detection on sorted cells. i) Sunburst charts showing relative frequencies of CD8+ T17^{lo}D_{exR}, CD8+ T17^{lo}D_{exR} and other CD8+ TD_{exR}, with host or donor genotype per sorted subset; NA indicates populations not meeting quality criteria for genotype annotation; each chart represents one patient. j) Quantification of CD8+ T17^{lo}D_{exR} and T17^{lo}D_{exR} frequencies and host cell percentage across groups (Paired t-test, not significant, n=3-5).

Extended Data Table 1

Host and donor skin T cells recovered per patient across timepoints in CD4 TD_RM and CD8 TD_RM clusters. Not sampled timepoints are indicated by na.

SKIN												
CD8+ TD _R M	Baseline		Tx		D14		D100		GVHD			
	ID	Host	Donor	Host	Donor	Host	Donor	Host	Donor	ID	Host	Donor
	P1	236	0	257	0	31	0	na	na	P6	236	0
	P2	96	0	95	0	na	na	na	na	P8	0	371
	P3	64	0	2	0	5	0	0	2	P9	2	164
	P4	37	0	11	0	5	0	3	105	P10	1	150
	P5	175	0	na	na	3	0	80	173	P11	1	8
	P6	199	0	na	na	46	2	na	na	P12	117	12
	P7	404	0	na	na	40	2	0	121			
	Baseline		Tx		D14		D100		GVHD			
CD4+ TD _R M	ID	Host	Donor	Host	Donor	Host	Donor	Host	Donor	ID	Host	Donor
	P1	90	0	45	0	6	0	na	na	P6	8	0
	P2	41	0	41	0	na	na	na	na	P8	0	111
	P3	35	0	1	0	4	0	0	0	P9	14	176
	P4	14	0	4	0	3	0	7	2	P10	0	60
	P5	86	0	na	na	2	0	86	17	P11	0	12
	P6	70	0	na	na	88	1	na	na	P12	42	10
	P7	422	0	na	na	88	0	0	452			
	Baseline		Tx		D14		D100		GVHD			
	ID	Host	Donor	Host	Donor	Host	Donor	Host	Donor	ID	Host	Donor

Supplementary Information

Supplementary tables

Table S1 – Extended clinical information of included patients

Table S2 – Overview of patient cohorts per analysis

Table S3 – Overview of antibodies and reagents used for CITEseq, flow cytometry and immunofluorescence

Table S4 – Immunofluorescence panels

Table S5 – Flow cytometry panels

Supplementary figures

Supplementary Figure S1 - High-resolution cell type annotation of skin and blood cells

Supplementary Figure S2 - Immunofluorescence supplementary information and isotype controls

Supplementary Figure S3 - Flow cytometry gating strategies and controls

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Author contributions: GS, LMG and FD conceptualized the study. GS supervised the study and provided funding. LT, DE 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. LH, EH, BR and LK performed experiments. LMG performed sample processing, bioinformatics analysis, experiments, formal analysis and data interpretation. LMG and FD 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), including reprocessed files (Cell Ranger v7.1.0) from our previously published dataset¹⁹ (GSE236264, Cell Ranger v3.0.2). Codes used for analyses performed in this paper, as well as the integrated anndata object corresponding to the UMAPs in Figure 1 and Supplementary Figure S1 will be made available on Zenodo.

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3 Extended discussion

High-throughput sequencing coupled with machine learning frameworks have contributed to reshaping our understanding of the immune systems as continua of cellular states. The manuscripts underlying this thesis show how inflammatory cues are integrated within tissue-specific immune niches to adapt cellular programs during immune challenge. They further demonstrate that these processes can support immune adaptation when properly coordinated but may contribute to immunopathology when timing or signaling context is disturbed. The central strength of this thesis is the generation and computational modeling of longitudinal sampled multimodal scRNA-seq datasets that capture cell states across distinct phases of inflammation. In a model of splenic stress erythropoiesis (Chapter 2.1), we profiled 46,765 splenic progenitors and myeloid cells across three genotypes and four time points. In the HSCT/aGVHD study (Chapter 2.2) we generated scRNA-seq transcriptomic and $\alpha\beta$ TCR-sequencing data from 124,698 CD45⁺ and CD45⁻ blood and skin cells from 12 patients across five time points. Whereas the introduction covered the immunological and computational foundation of the thesis, the discussion will extend this framework by integrating relevant clinical aspects, identifying remaining research gaps, and defining the specific contributions of this work. In addition, the discussion will incorporate unpublished data and complementary lines of evidence that were not fully explored in the underlying manuscripts.

In the first manuscript (Chapter 2.1) presented in this thesis, “**Myeloid type I interferon sensing boosts splenic stress erythropoiesis**” we investigated how inflammatory signals are temporally coordinated within the splenic erythropoietic niche to support stress erythropoiesis when steady-state bone marrow erythropoiesis is compromised.

Anemia of inflammation and regulation of systemic iron homeostasis

Anemia of inflammation reflects a fundamental trade-off between host defense and erythroid output. Anemia is clinically defined by a reduction in hemoglobin concentration below the level required to meet tissue oxygen demands (Braat et al., 2024). It represents one of the most common hematological disorders worldwide. The Global Burden of Disease Study estimated that 1.92 billion people were affected by anemia in 2021, corresponding to approximately 24.3% of the global population (GBD 2021 Anaemia Collaborators, 2023). The causes of anemia are highly heterogeneous and include nutritional deficiencies, inherited hemoglobin disorders, infections, chronic kidney disease, malignancy, inflammatory diseases, and socioeconomic determinants that influence exposure, diagnosis, and access to treatment (Atkinson et al., 2025). Anemia of inflammation is a clinically significant complication of chronic inflammatory disease and estimated to be the cause of up to 40% of all anemias worldwide (Weiss et al., 2019). Like iron-deficiency anemia, it is characterized by hypoferrremia. However,

the underlying iron defect is fundamentally different. In iron-deficiency anemia, total body iron stores are depleted, whereas in anemia of inflammation, iron is sequestered within macrophages and hepatocytes, resulting in hyperferritinemia (Ganz, 2019). At the physiological level, IL-6 restricts iron mobilization by inducing hepatic production of hepcidin (HAMP). Hepcidin binds to the iron exporter ferroportin (SLC40A1), which is expressed by iron-recycling macrophages, duodenal enterocytes, and hepatocytes, thereby promoting ferroportin internalization and degradation (Nemeth et al., 2004). Evolutionarily, iron withdrawal during the acute phase of infectious diseases represents a conserved host-defense mechanism that limits the availability of iron required for the growth and virulence of many bacteria, fungi, and parasites (Ganz & Nemeth, 2015).

Type I IFN sensing license red pulp macrophage programs in monocytes

A core finding of the first study presented in this thesis was that recruited monocytes required IFN-I sensing already by day one after CpG challenge to acquire an RPM-like transcriptional state, including upregulation of *Spic* and iron-recycling programs. We also noted a balance shift of IFN-I to IFN-II response genes on day one in *Ifnar1^{fl/fl} LysM^{cre/+}* mice. Although *Ifnar1^{fl/fl} LysM^{cre/+}* mice accumulated increased numbers of RPMs by day six, these cells showed reduced expression of RPM maturity markers.

IFN-I are important mediators of host defense against viral and bacterial infection (McNab et al., 2015). However, they exert pleiotropic effects on many immune cells, and their mode of action is highly context dependent (Boehmer & Zanoni, 2025). IFN-I signaling has been linked to increased susceptibility to several pathogens that infect phagocytes, including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Brucella abortus*, and *Yersinia pestis*, while IFN-II signaling promotes antimicrobial macrophage activation and is generally required for efficient control and clearance of intracellular pathogens (Dhariwala et al., 2017; Rayamajhi et al., 2010; Teles et al., 2013; G. Zhang et al., 2026).

Increased susceptibility is often discussed in the context of activation of antiviral programs that are mostly ineffective against intracellular bacteria and parasites and may antagonize IFN-II macrophage activation. However, in the RPM context, the antagonistic effects of IFN-I on IFN-II programs may be important for inducing iron-recycling programs in monocytes. Importantly, the core RPM transcription factor *Spic* is not exclusively expressed by RPMs but can also be induced in monocytes and macrophages across tissues independently of heme in response to inflammation. Alam et al. showed *in vivo* that TLR4 dependent *Spic* expression is associated with suppression of pro-inflammatory cytokine production and with macrophage iron efflux (Alam et al., 2020). While these functions may be important during the resolution phase of inflammation, they can be detrimental to host defense against pathogens. The authors further

showed that IFN-II, but not IFN-I, suppresses *Spic* expression, thereby regulating the balance between inflammatory macrophage activation and iron-handling programs.

However, expression of *Spic* alone in monocytes and macrophages is not sufficient to establish a full RPM phenotype (Akilesh et al., 2019). Akilesh et al. showed that *In vivo* stimulation of TLR7 or TLR9 induces *Spic* expression in bone marrow and splenic monocytes, but the resulting macrophages do not acquire full RPM marker gene expression. Instead, they display a highly inflammatory and hemophagocytic phenotype. Notably, these hemophagocytic *Spic* expressing macrophages show reduced surface expression of VCAM1 and CD64, resembling the phenotype we observed in *Ifnar1^{fl/fl} LysM^{cre/+}* RPMs. These findings highlight the importance of IFN-I in shaping macrophage function and raise the possibility that monocyte priming toward an RPM-like phenotype may begin already in the bone marrow. They further support the broader concept that TLR signaling can initiate RPM-associated transcriptional programs, while additional inflammatory cues such as IFN-I are required for full RPM adaptation during inflammation.

Erythroid progenitors display a complex inflammatory response signature

A surprising finding of our investigation was that stress erythroid progenitors in the spleen display an inflammatory stress response centered on transcriptional activation of MyD88, AP-1, and STAT family transcription factors. The observation that stress erythropoiesis is a substantial part of immune responses rather than a bystander effect was introduced by Paulson and colleagues (Paulson, Ruan, et al., 2020).

In our data, Myd88 expression increased on day one and day three in erythroid progenitors, with peak expression at the CFU-E stage. This signal was comparable between *Ifnar1^{fl/fl} LysM^{+/+}* and *Ifnar1^{fl/fl} LysM^{cre/+}* mice and associated with metabolic rewiring of stress erythroid progenitors. MYD88 is typically activated downstream of either IL-1 family cytokine receptors or TLRs. Recent findings showed that human and mouse red blood cells express TLR9 both intracellularly and at the cell surface (Hotz et al., 2018; L. K. M. Lam et al., 2021). At the cell surface, DNA binding disrupts the protective CD47–SIRPα interaction and thereby promotes erythrophagocytosis. However, the role of endosomal TLR9 in erythroid cells remains less clear. Although we did not detect TLR transcripts in the erythroid lineage, Unc93b1, the endosomal trafficking chaperone required for nucleic acid-sensing TLRs, was transcribed at the earliest erythroid progenitor states (Y.-M. Kim et al., 2008). Although hematopoietic stem and progenitor cells are well established to express TLRs, and TLR stimulation can promote proliferation and lineage specification, erythroid- and megakaryocyte-committed progenitors are generally not considered to express TLRs (Nagai et al., 2006). Accordingly, TLR activation is thought to preferentially bias hematopoiesis toward myelopoiesis and granulopoiesis rather

than erythroid differentiation. However, the data presented in this manuscript, together with recent evidence that red blood cells express TLR9, suggest that the potential capacity of stress erythroid progenitors to incorporate TLR signals warrants further investigation.

On day three, early erythroid progenitors showed induction of AP-1 complex genes (*Atf3*, *Jun*, *Junb*, *Jund*, *Fos*, *Fosb*), which was associated with predicted sensing of TNF derived from classical monocytes. The AP-1 and TNF expression was significantly downregulated in *Ifnar1^{fl/fl}* *LysM^{cre/+}* mice. In contrast to bone marrow erythropoiesis, where TNF is generally considered suppressive, splenic myeloid-derived TNF has been associated with increased erythroblast output during stress erythropoiesis (Bennett et al., 2019). AP-1 activation is linked to pro-survival and proliferative signaling downstream of ERK/MAPK pathways and may therefore contribute to stress erythroid progenitor expansion. However, AP-1 is also a rapid, transient stress-response program, and the temporal resolution of our sampling may be insufficient to capture its downstream regulatory consequences within the erythroid trajectory.

On day one and day three, intermediate monocytes with an RPM-like transcriptional phenotype expressed *Ccl2*, *Cxcl10*, and *Cxcl9* chemokines and expression were dependent on myeloid IFN-I sensing. Interestingly, the chemokines were predicted to bind to the atypical chemokine receptor 1 (ACKR1), which is expressed by erythroblasts and mature red blood cells. ACKR1 - also known as DARC or the Duffy - binds multiple inflammatory chemokines but lacks canonical G protein-coupled intracellular signaling (Comerford & McColl, 2024). Rather than functioning as a classical chemotactic receptor, ACKR1 is thought to regulate chemokine availability, buffering, and spatial presentation. ACKR1 is also clinically relevant as the Duffy blood group antigen and serves as an erythrocyte entry receptor for *Plasmodium vivax*. Loss of erythroid ACKR1 expression, referred to as the Duffy-null phenotype, is common in individuals of African ancestry and confers protection against malaria. However, ACKR1 deficiency is also associated with neutropenia and it has been shown that erythroid ACKR1 is regulating splenic extramedullary hematopoiesis (Duchene et al., 2017). We therefore speculated that erythroid ACKR1 may regulate chemokine gradients during stress erythropoiesis. In preliminary collaborative experiments with Antal Rot, erythroid-specific ACKR1 deficiency reduced erythroblast output to a degree comparable to myeloid IFNAR deficiency. However, financial constraints and loss of access to the mouse model prevented further validation, and the data were therefore not mature enough for inclusion in the manuscript. This leaves erythroid ACKR1 as an interesting but unresolved regulator of chemokine organization within the splenic stress erythropoietic niche.

We further showed that erythroid progenitors display an interferon-response program, which was reduced by day three in *Ifnar1^{fl/fl}* *LysM^{cre/+}* mice. Compromised interferon sensing was

accompanied by increased expression of EPO response genes in erythroblasts, including erythroferrone, which represses hepcidin and thereby promotes iron availability. These findings suggest that altered erythroid niche signaling downstream of myeloid interferon sensing may contribute to systemic iron regulation. Paradoxically, erythroid output was significantly reduced in *Ifnar1^{fl/fl} LysM^{cre/+}* mice. It is therefore intriguing to speculate that interferon signaling cooperates with canonical JAK–STAT pathways of erythroid development to support efficient stress progenitor expansion during inflammation but must also be tightly coordinated to not prematurely commit to terminal erythroid differentiation.

Translational aspects of mouse stress erythropoiesis

A major limitation in translating murine stress erythropoiesis to humans is the anatomical divergence of adult erythroid niches. In mice, inflammatory or anemic stress induces a specialized extramedullary erythropoietic response, most prominently in the spleen. In adult humans, however, erythropoiesis is predominantly restricted to the bone marrow and stress erythroid pathways analogous to those defined in murine models have not been confidently established *in vivo* (Paulson, Hariharan, et al., 2020). Nevertheless, our findings may have implications for inflammatory cytopenias such as secondary hemophagocytic lymphohistiocytosis (sHLH), a hyperinflammatory syndrome in which infections, malignancies, or autoimmune diseases trigger excessive T cell and macrophage activation, resulting in cytokine storm, hemophagocytosis, hyperferritinemia, splenomegaly, and multilineage cytopenias (Henter, 2025). Current therapeutic approaches focus on treatment of the underlying trigger and rapid suppression of hyperinflammation. First-line treatment options are administration of etoposide and glucocorticoids together with more condition specific drugs including inhibitors of IL-1, IFN- γ , and Janus kinases. Such treatment strategies focus on disrupting the inflammatory loop between cytotoxic lymphocytes and macrophages, which fuels excessive cytokine production, tissue damage, hemophagocytosis, and inflammasome associated pyroptosis (Andersson, 2021). Our findings suggest that IFN-I-dependent pathways may have therapeutic potential in selected forms of sHLH by modulating macrophage activation states toward more regulatory and tissue-supportive phenotypes. However, given the pro-inflammatory potential of IFN-I signaling, such approaches would likely require targeted modulation of specific downstream IFNAR-dependent pathways rather than broad activation of the IFN-I axis.

Conclusions and outlook

In our manuscript, we present a high-resolution single-cell RNA-sequencing model of the splenic erythroid niche that captures progenitor and myeloid cell dynamics at steady state and

during inflammation. A pressing question in the field is how stress erythropoiesis integrates inflammatory signals that usually suppress bone marrow erythropoiesis.

While diffusion pseudotime analysis identified activated pathways in erythroid progenitors, we did not directly compare this signature with bone marrow hematopoiesis. Such a comparison would be important because it remains unclear whether splenic hematopoiesis is already primed under steady-state conditions, or whether this process depends entirely on the expansion of dedicated stress erythroid progenitors derived from primed HSCs. Similarly, although the model faithfully captures the expression of stress erythroid progenitor markers, we could not define a regulatory mechanism explaining why stress erythroid progenitors thrive under inflammatory conditions. In this regard, sampling one day after CpG stimulation may already be too late to capture the earliest stages of BFU-E priming and expansion. Conversely, given that virtually all erythroid lineage cells displayed a cytokine-response profile, it would also be interesting to assess whether splenic steady-state progenitors can adapt to stress erythropoiesis independently of BMP4-primed HSC expansion.

A core strength of the study was the combined analysis of RPMs and the erythroid lineage. However, it is important to note that RPMs were not sampled without bias. Owing to their erythrophagocytic activity and their role as erythroblastic island cells, RPMs are particularly prone to exclusion during FACS sorting and computational filtering, as they can resemble doublets. Although we have no evidence that this biased our data in a harmful way, it remains an important technical limitation. In addition, we did not detect other splenic macrophage subsets, likely due to their low abundance or difficulties in tissue digestion and recovery during sorting. While scRNA-seq provided valuable insights, it remains a transcriptome-based approach that infers signaling cascades and cellular states from gene-expression profiles. Although we experimentally validated our main findings, additional measurements of cytokine profiles, iron stores, and STAT phosphorylation states would have further strengthened our mechanistic interpretation.

Erythrocytes have traditionally been viewed as the non-leukocytic arm of the myeloid compartment. However, dedicated work over the last decade has increasingly shown that erythroid cells are not passive bystanders of the immune system but can actively shape immune responses. With our study, we aim to contribute to this emerging concept and further support the recognition of the erythroid lineage as an active participant in immune regulation.

In the second manuscript (Chapter 2.2) presented in this thesis, **“Disruption of skin homeostasis reveals divergent reconstitution dynamics of human skin tissue-resident memory T cell subsets”** we investigated how host- and donor-derived immune cells reconstitute the human skin and blood compartments after HSCT and during aGVHD. Because

the broader implications of this manuscript for TDRM biology and therapeutic translation are discussed extensively in the thesis of Laura Marie Gail, the present thesis focuses instead on the computational aspects of the study.

Modeling human transplantation dynamics using multimodal scRNA-seq data

Most insights into human skin TD_RM biology have been derived from mouse models or inferred from human blood samples. Although blood profiling is important for biomarker discovery in aGVHD, it only indirectly approximates the biology of TD_RM after HSCT. Longitudinal sampling of paired blood and skin therefore provided a unique opportunity to analyze immune reconstitution across compartments. In mouse models, lineage tracing can be used to distinguish circulating blood T cells (TD_B) from cells that acquire tissue residency (TD_RM) or re-enter circulation through retrograde migration (TD_{exR}). Since such approaches are not feasible in human studies, we used $\alpha\beta$ TCR sequencing to track clonally related T cells across blood and skin and to infer their relationship between tissues (Pai & Satpathy, 2021). T cell clones are defined by exact matching of the CDR sequences at the nucleotide or amino acid level. More broadly, public TCR clones binding the same antigen can also be inferred using distance-based receptor-similarity metrics that tolerate sequence substitutions, or by neural-network-based approaches that model antigen receptor similarity. However, these approaches rely either on the assumption that T cells expanded across different patients recognize the same antigen and therefore display convergent receptor features, or on sufficiently large cohorts to detect robust receptor-similarity patterns. Since neither condition was met in our study, cross-patient analysis of clonal expansion during aGVHD remained limited in its generalizability.

To study host–donor chimerism in the skin, individual cells first had to be assigned to either host or donor origin. In transplantation biology, this can be achieved in sex-mismatched donor–recipient pairs by using sex specific gene expression. However, this approach is inherently limited, because it depends on the availability of sex-mismatched transplantation settings and cannot be generalized to all patients. An alternative approach is to use genetic variation to distinguish host- from donor-derived cells. In single-cell analysis, variant-based demultiplexing is commonly used to separate pooled patient samples, conceptually similar to experimental multiplexing with hashtag antibodies or lipid-conjugated oligonucleotide barcodes. Instead of relying on externally introduced sample labels, these methods exploit single nucleotide polymorphism (SNP) between individuals (Fu et al., 2025). We showed that the same principle can be used to assign each cell to either the host or donor genotype, enabling host–donor chimerism to be resolved directly at single-cell resolution. This approach has since attracted interest from several other research groups.

The core strength of the manuscript is the cross-tissue integration of the single-cell atlas and the high-resolution annotation of immune and non-immune cell types. This was enabled by deep generative modeling with scVI, which allowed technical and patient-specific covariates to be incorporated and variation unrelated to the biological questions of interest to be reduced. Although this framework allows continuous and categorical covariates to be incorporated flexibly, it could not overcome the limitations imposed by cross-sectional sampling of HSCT and aGVHD samples. This partly restricted the use of pseudotime-based approaches, because patient-specific variability could not be fully separated from condition-specific variability when patients were not shared across groups.

Together, this unique dataset and its computational modeling framework provide a valuable resource for the field and enabled us to uncover new aspects of human TD_RM biology after HSCT and during aGVHD.

Novel insights into human TD_RM biology during HSCT and aGVHD

Previous studies using X/Y-FISH in situ genotyping in sex-mismatched donor–recipient pairs showed that host-derived skin T cells, including TD_RM-like populations, can persist for years after myeloablative conditioning, with some GVHD patients retaining more than 50% host-derived skin T cells. (Strobl et al., 2020). In our SNP-demultiplexed single-cell dataset, the blood compartment was almost completely donor-reconstituted shortly after transplantation, whereas reconstitution of skin leukocyte subsets was delayed. However, we detected a lower proportion of host-derived TD_RM after transplantation and during GVHD than reported in previous studies, although the extent of host persistence varied substantially between patients. Whether this discrepancy reflects the limited cohort size, methodological differences in host–donor assignment, differences in the analyzed cell populations, or true biological variation remains unclear.

The $\alpha\beta$ TCR clonotype data were not sufficient to robustly identify public clones shared across patients, and longitudinal persistence of individual skin clones within patients was sparse. However, we detected multiple clonally matched T cells shared between blood and skin within individual patients after HSCT, as well as during aGVHD. These shared clones showed a strong overlap between blood effector memory T cells (TD_B) and skin TD_RM but rarely overlap with blood central memory or naïve T cells (TS_B). This strongly suggests that most donor TD_RM are derived from TD_B cells, a possibility that remains actively debated in the field.

Our data showed that, after transplantation, donor-derived cells efficiently reconstituted T₁DRM, whereas CD8⁺ T₁₇DRM remained selectively reduced during HSCT and aGVHD, with

recovery of this compartment becoming detectable only in late post-transplant settings and after aGVHD resolution.

One possible explanation is that the post-transplant skin niche favors T₁D_RM polarization, recruitment and maintenance while failing to provide signals required for CD8⁺ T₁₇D_RM reconstitution. This interpretation is consistent with our observation that GVHD lesions displayed strong IFN-I and IFN-II response signatures and induction of CXCR3 ligands such as CXCL9, CXCL10 and CXCL11. At the same time, IL15 expression was elevated in fibroblasts already in post-HSCT skin and increased further during GVHD, whereas TD_RM showed marked downregulation of IL7R, suggesting reduced IL-7 responsiveness despite local availability of the IL15. Thus, radio-chemotherapy-induced tissue damage and DAMP release may establish a sterile inflammatory skin niche that favors T₁D_RM polarization while limiting efficient T₁₇D_RM reconstitution.

Murine CD8⁺ T₁D_RM and T₁₇D_RM are generated through partly distinct developmental programs. In mouse skin, T₁D_RM generated after viral infection depend on a T-bet–Hobit–IL-15 axis, whereas T₁₇D_RM induced after *Staphylococcus epidermidis* colonization depend on an ICOS–c-Maf–IL-7 axis and develop independently of the T₁D_RM program. This distinction cannot be transferred directly to humans, but our data supports partial conservation. Human CD8⁺ T₁₇D_RM were enriched for T17-associated regulatory programs including STAT3, AHR and BATF, while displaying reduced cytotoxic and antiviral genes compared with other TD_RM. In contrast to murine skin, where ICOS has been linked more selectively to CD8⁺ T₁₇D_RM maintenance, our data showed broad ICOS induction in human CD8⁺ TD_RM during aGVHD at the protein level.

This could be interpreted in two ways. First, it may indicate divergent regulatory mechanisms between murine and human CD8⁺ TD_RM, in which ICOS is not selectively linked to human CD8⁺ T₁₇D_RM maintenance but instead reflects broader activation role in both subsets. Second, it may suggest that CD8⁺ T₁₇D_RM states are not fully lost after HSCT or during aGVHD but become redirected by the inflammatory tissue environment. Given their potential plasticity, CD8⁺ T₁₇D_RM may acquire IFN-II expression, shifting toward a T₁D_RM-like phenotype without fully completing this transition. In this model, the apparent reduction of CD8⁺ T₁₇D_RM could partly reflect state deviation along a continuous T₁₇D_RM–T₁D_RM axis rather than complete loss of the underlying population. We further found that blood CD8⁺ TD_{exR} mirrored the TD_RM subset skewing observed in skin and may therefore serve as a prospective biomarker. However, future studies should also address whether the HSCT inflammatory blood environment programs already suppress T17-associated programs in TD_B before skin entry.

Transplant conditioning as a determinant of TD_RM reconstitution

Our manuscript provided new insights into human T_DR_M biology but also raises important questions about the mechanisms that regulate CD8⁺ T₁₇D_RM recovery after HSCT and during aGVHD. The discussion so far has focused on the possibility that inflammatory blood and skin microenvironments suppress or impairs efficient CD8⁺ T₁₇D_RM reconstitution. However, inflammatory cytokine signaling alone may not fully explain the selective loss of this compartment. Additional transplantation related regimens could cooperate with inflammation to suppress T17-associated programs or prevent their stable maintenance.

Development of CD4⁺ T₁₇D_RM is closely linked to signals from commensal skin microbiota in mouse models, and potentially also in humans. During the neutropenic phase after HSCT, patients receive systemic antimicrobial prophylaxis that can substantially perturb skin microbial communities. In previous work, we showed disturbed microbial skin recolonization after allogeneic stem cell transfer, including reduced bacterial colonization after conditioning and *Staphylococcus* overgrowth in patients who developed cutaneous aGVHD (Bayer et al., 2022). Considering our current findings, microbial dysbiosis during HSCT may impair the reconstitution of donor-derived CD8⁺ T₁₇D_RM and prime exacerbation phase later exacerbation phase during aGVHD.

Finally, post-transplant patients receive intensive immunosuppressive treatment, which may further interfere with CD8⁺ T₁₇D_RM development or maintenance. In our dataset, IL7 was produced within the skin, particularly by fibroblasts, whereas its receptor IL7R/CD127 was downregulated on T_DR_M. This raises the possibility that post-HSCT immunosuppressive regimens or other treatment-associated factors suppress IL7R expression or interfere with IL-7 responsiveness, thereby limiting efficient T₁₇D_RM reconstitution. However, this remains speculative, as direct experimental evidence linking specific immunosuppressive drugs to impaired CD8⁺ T₁₇D_RM development is currently lacking in our study.

Conclusions and outlook

Collectively, our manuscripts provide new insights into human T_DR_M biology with important clinical implications. However, important questions remain unresolved, particularly regarding the mechanisms underlying impaired T₁₇D_RM formation after HSCT. To address this, we are currently investigating fibroblast heterogeneity in greater detail by integrating our data with a published fibroblast atlas, aiming to identify interactions between fibroblasts and T cells that may hinder T₁₇D_RM formation. Furthermore, although we successfully used TCR sequencing for clonal tracing, antigen specificity was not determined in our manuscript. This limitation has led to a second project in which we aim to identify the antigen specificity of expanded T cell clones during aGVHD and assess whether these clones are associated with pathogen reactivation or alloantigen recognition.

4 Materials & Methods

Detailed information on the materials and methods used in this thesis is provided in the corresponding Materials and Methods sections of the individual manuscripts.

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- Zou, D., Li, X. C., & Chen, W. (2025). Beyond T-cell subsets: stemness and adaptation redefining immunity and immunotherapy. *Cellular & Molecular Immunology*, 22(9), 957–974.
- Zou, Y., & Yuan, H. (2021). The Pathogenic Role of CD4(+) Tissue-Resident Memory T Cells Bearing T Follicular Helper-Like Phenotype in Pemphigus Lesions. *J Invest Dermatol*, 141, 2141–2150.

Appendix

No separate appendix was submitted as part of this thesis. Relevant information was retained within the chapters of the two included manuscripts to preserve clarity and readability.

Curriculum vita

FLORIAN DECKERT

Computational Immunology | Biomedical data analysis | Applied AI/ML

 ORCID  GitHub  LinkedIn

EDUCATION

Predoctoral Fellow, Immunology, Medical University of Vienna, Austria since 09/2019
[Sylvia Knapp Group](#), Research Division Infection Biology, Department of Medicine I
Research Focus: Splenic stress erythropoiesis, role of alveolar macrophages in lung disease, and mRNA editing during lung infection and colitis

[Georg Stary Group](#), Department of Dermatology
Research Focus: Skin-resident memory T cell homeostasis following hematopoietic stem cell transplant and during graft versus host disease

Center for Molecular Medicine (CeMM)
Ph.D. Program

Paternity leave
Full break from 09/2022 - 10/2022 and 12/2025 - 01/2026

M.Sc., Biology, Ludwig-Maximilians-University of Munich (LMU), Germany 10/2015 - 09/2018
Thesis: Integrated analysis of mouse neural stem cells with DNMT3A overgrowth mutations

B.Sc., Biotechnology, Weihenstephan University of Applied Sciences, Germany 09/2009 - 09/2014
Thesis: High-throughput sequencing of sequential permuted gene libraries

Advanced Technical Certificate, Technical High School Bad Tölz, Germany 09/2008 - 07/2009

Dual Pharmaceutical Vocational Training, VERLA-PHARM, Germany 09/2005 - 06/2008

RESEARCH EXPERIENCE

University of California Los Angeles, USA 05/2017 - 05/2018
M.Sc. Thesis Project, Department of Human Genetics, Prof. Fan
Multimodal data analysis of mouse neuronal stem cells with CRISPR/Cas9 induced DNMT3A mutations (Tatton-Brown-Rahman Syndrome)

Ludwig-Maximilians-University Munich, Germany 10/2016 - 02/2017
M.Sc. Research Internship, Department of Human Genomics, Prof. Enard
Nanopore sequencing human and macaque induced pluripotent stem cells

Weizmann Institute of Science, Israel 08/2016 - 09/2016
M.Sc. Research Internship, Department of Biological Regulation, Dr. Ulitsky
Epigenetic regulation of imprinted genes by Chd2 in mouse embryonic bodies

Scientific Assistant, Max-Planck-Institute of Biochemistry, Germany 11/2014 - 09/2015
Computational Biology Group, Dr. Habermann
Research Focus: Denovo assembly of Drosophila genomes

GeneArt AG, Germany 08/2013 - 02/2014
B.Sc. Thesis Project, Department of Research and Development
Implementation of high-throughput sequencing for quality control of oligonucleotide synthesis products

Mbeya Medical Research Center, Tanzania 10/2011 - 02/2012
B.Sc. Practical Semester, Tuberculosis Laboratory
Detection and drug susceptibility testing of Mycobacterium tuberculosis in sputum samples

AWARDS & FUNDING

Immunology Research Cluster (IRC) Travel Grant Poster presentation, 19 th International Congress of Immunology (IUIS 2025, Vienna) Funding amount: 500 Euro	2025
Immunology Research Cluster (IRC) Travel Grant Oral presentation, 7 th European Congress of Immunology (ECI 2024, Dublin) Funding amount: 900 Euro	2024
Visiting Graduate Researcher (VGRs) Stipend UCLA Stipend (University of California Los Angeles, USA) Funding amount: 5.000 Euro	2017
PROSA LMU Travel Grant funded by German Academic Exchange Service (DAAD) Research internship abroad (University of California Los Angeles, USA) Funding amount: 1.800 Euro	2017
PROMOS LMU Travel Grant funded by German Academic Exchange Service (DAAD) Research internship abroad (Weizmann Institute of Science, Israel) Funding Amount: 500 Euro	2016

SKILLS

Data science setup	Python, R, Bash, Conda, JupyterLab, GitHub
Systems	Slurm HPC, Linux, Windows
NGS technologies	scRNA-seq, bulk RNA-seq, bulk ATAC-seq, ChIP-seq, spatial transcriptomics
Structural analysis	TCR clonotyping, AlphaFold (basics)
Previous experience	NGS-library preparation and cell culture systems
People skills	Project management, event organization, public speaking

EXTRACURRICULAR ACTIVITIES

CeMM Ph.D. Representative	2019 - 2020
KinderuniWien, Vom Gen, zur Zelle, Organismus	2020
Memberships	
European Macrophage and Dendritic cell Society (EMDS)	
Austrian Society for Allergology and Immunology (ÖGAI)	

CONFERENCES & TALKS

Poster presentation, 19 th International Congress of Immunology, IUIS 2025, Vienna	2025
Oral presentation, 7 th European Congress of Immunology, Dublin	2024
Oral presentation, European Society of Hematology 3 rd Translational Research Conference, Paris	2023

PEER REVIEWED PUBLICATIONS

Gawish R*, Varada R, Deckert F, Hladik A, Steinbichl L, Cimatti L, Milanovic K, Jain M, Torgasheva N, Tanzer A, De Paepe K, Van de Wiele T, Hausmann B, Lang M, Pechhacker M, Ibrahim N, De Vries I, Brostjan C, Sixt M, Gasche C, Boon Ö, Berry D, Jantsch MF, Pereira FC, and Vesely C

Filamin A editing in myeloid cells reduces intestinal inflammation and protects from colitis
Journal of Experimental Medicine 2025 Sep 1;222 (9):e20240109

Farhat A, Radhouani M, Deckert F, Zahalka S, Pimenov L, Fokina A, Hakobyan A, Oberndorfer F, Brösamlen J, Hladik A, Lakovits K, Meng F, Quattrone F, Boon L, Vesely C,

Starkl P, Boucheron N, Menche J, Van der Veeke J, Ellmeier W, Gorki AD, Campbell C, Gawish R, Knapp S

An aging bone marrow exacerbates lung fibrosis by fueling profibrotic macrophage persistence

Science Immunology 2025 Mar 28; 10(105):eadk5041

Stary V, Pandey RV, List J, Kleissl L, Deckert F, Kabiljo J, Laengle J, Gerakopoulos V, Oehler R, Watzke L, Farlik M, Lukowski SW, Vogt AB, Stary G, Stockinger H, Bergmann M, Pilat N

Dysfunctional tumor-infiltrating $\gamma\delta 1+$ T lymphocytes in microsatellite-stable colorectal cancer

Nature Communications 2024 Aug 13;15(1):6949

Bayer N, Hausmann B, Pandey RV, Deckert F, Gail LM, Strobl J, Pjevac P, Krall C, Unterluggauer L, Redl A, Bachmayr V, Kleissl L, Nehr M, Kirkegaard R, Makristathis A, Watzenboeck ML, Nica R, Staud C, Hammerl L, Wohlfarth P, Ecker RC, Knapp S, Rabitsch W, Berry D, Stary G

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

Leukemia 2022 Nov;36(11):2705-2714

Zahalka S, Starkl P, Watzenboeck ML, Farhat A, Radhouani M, Deckert F, Hladik A, Lakovits K, Oberndorfer F, Lassnig C, Strobel B, Klavins K, Matsushita M, Sanin DE, Grzes KM, Pearce EJ, Gorki AD*, Knapp S*

(* equal contribution)

Trained immunity of alveolar macrophages requires metabolic rewiring and type 1 interferon signaling

Mucosal Immunology 2022 May;15(5):896-907

MANUSCRIPTS IN REVISION

Gail LM*, Deckert F*, Thébault L, Dingelmaier-Hovorka R, Strobl J, Eskandari D, Kleissl L, Reininger B, Mayerhofer C, Gabriel A, Freystätter C, Shaw L, Rüsing L, Cserna J, Weninger W, Farlik M, Rabitsch W,

Stary G

(* equal contribution)

Disruption of skin homeostasis reveals divergent reconstitution dynamics of human skin tissue-resident memory T cell subsets

Under revision at Nature Immunology

Fokina A, Pimenov L, Hladik A, Lakovits K, Farhat A, Deckert F, Radhouani M, Broesamlen J, Meng F, Gawish R & Knapp S

Early and late pregnancy differentially impact maternal alveolar macrophages' function and immunity to SARS-CoV-2 infection

Under revision at Mucosal Immunology

Winkler F, Fischer C, Gail LM, Deckert F, Kleissl L, Krausgruber T, Brazdilova K, González AA, Stary G, Strobl J

Virus-specific resident memory T cell networks sustain immunity in human oral mucosa

Under revision at Mucosal Immunology

MANUSCRIPTS SUBMITTED OR UNDER REVIEW

Deckert F, Hladik A, Lakovits K, Farhat A, Pimenov L, Meng F, Fokina, Quattrone F, Gorki AD, Gawish R*, Knapp S*

(* equal contribution)

Myeloid type I interferon sensing boost splenic stress erythropoiesis

Submitted to Blood

Ntayi M, Knap K, Deckert F, Chrapla K, González AA, Bauer W, Krausgruber T, Grabmeier-Pfistershammer K, Saluzzo S*, Stary G*

(* equal contribution)

Spatially organized cutaneous immunity orchestrates antiviral responses during acute MPOX infection

Submitted to Nature Immunology

Pimenov L, Deckert F, Fokina A, Farhat A, Radhouani M, Broesamlen J, Fanzhe M, Hladik A, Lakovits K, Salzer I, Schicker K, Repic A, Starkl P, Gawish R, Knapp S

Passenger mutations in epithelial cells can autonomously determine outcomes of respiratory viral infections

Submitted

MANUSCRIPTS IN PREPARATION

Deckert F*, Fanzhe M*, Vesely C, Horstmeier HL, Hladik A, Lakovits K, Jantsch MF, Knapp S, Gawish R

(* equal contribution)

Adar2 is a novel regulator of alveolar macrophage antiviral effector functions during SARS-CoV-2 infection