Identification of novel CD8+ T cell homing markers in experimental murine and human acute GvHD

Ph.D thesis

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1. Background

Acute graft-versus-host disease (aGvHD) is a common, life-threatening side effect of allogeneic hematopoietic stem cell transplantation (aHSCT). Acute GvHD is induced by various graft-derived immunocompetent cells, most prominently graft-derived naïve CD8+ T cells. In aGvHD, CD8+ T cells exert cytotoxic effects on a limited set of organs of the aHSCT recipient, typically the skin and gut, occasionally the liver.

Interestingly, the skin and the gut represent the best known, but still not completely understood targets of CD8+ T cell homing in the human body. Hence, by coincidence, aHSCT patients diagnosed with cutaneous and/or gastrointestinal aGvHD provide a unique opportunity to perform in-depth, comparative studies on human CD8+ T cells activated simultaneously, under the same conditions, but pre-programmed to home to two distinct organ targets, the gut and skin.

2. Objectives

The aim of my Ph.D thesis was to identify novel biomarkers of skin- and gut-homing homing CD8+ T cell subsets by comparing them in the context of aGvHD, and to test whether they could be of any prognostic or diagnostic relevance. Studies were carried out both on a mouse experimental model and on clinical samples of human aGvHD patients.

1. Our first objective was to develop a novel transgenic aGvHD mouse model capable of inducing robust and reproducible aGvHD in all typical human target organs based on a purely monoclonal CD8+ T cell response, developed against a single, defined, and well-known minor histocompatibility antigen (miHA) serving as mismatch between the aHSCT donor and recipient. This rationale of this design was to allow direct comparison of CD8+ T cell homing subsets in cutaneous and gastrointestinal aGvHD in a pure experimental system.

2. Second we sought to analyse blood samples of human aGvHD patients and healthy volunteers to describe and study novel human CD8+ T cell homing biomarkers important in tissue specific migration or/and in aGvHD and assess their prognostic and diagnostic potential.

3. Methods

Methods used in mouse model experiments

Used mouse strains:

Name	Abbreviation	Modification
C57BL/6-Tg(CAG-OVA) 916Jen/J	Act-mOVA	Ovalbumin expression in mice of this strain is detected immunohistochemically on the surfaces of cells of all organs and functions in skin-graft experiments as a minor histocompatibility antigen that triggers graft rejection by a mechanism involving both CD4 and CD8 T lymphocytes. This mutant mouse strain represents a model that may be useful in studies of adoptive transfer and graft rejection as source for tissue and cells expressing a defined minor histocompatibility antigen.
C57BL/6-Tg(TcraTcrb) 1100Mjb/J	OT-I	These mice contain transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes. The transgenic T cell receptor was designed to recognize ovalbumin peptide residues 257-264 (OVA257-264) in the context of H2Kb (CD8 co-receptor interaction with MHC class I). This results in MHC class I-restricted, ovalbumin-specific, CD8+ T cells (OT-I cells). That is, the CD8 T cells of this mouse primarily recognize OVA257-264 when presented by the MHC I molecule. Immune response dynamics can be studied by in vivo adoptive transfer or in vitro co-culture with cells transgenic for ovalbumin or by direct administration of ovalbumin.
B6.SJL-Ptprca Pepcb /BoyJ	CD45.1	This C57BL/6 congenic strain is used widely in transplant studies because it carries the differential Ptprca pan leukocyte marker commonly known as CD45.1 or Ly5.1. Wild-type C57BL/6 inbred mice express the Ptprcb (CD45.2 or Ly5.2) allele.
C57BL/6-Tg(UBC-GFP)30Scha/J	UBC-GFP	These transgenic mice express enhanced Green Fluorescent Protein (GFP) under the direction of the human ubiqutin C promoter in all tissues examined. Certain hematapoetic cell types display distinct expression levels of GFP, allowing identification of different cells types by FACS analysis. This mutant mouse strain represents a useful tool in studies related to hematopoetic cell differentiation and in vivo tracking of leukocytes.

OT-I → OVA acute GvHD model

CD8+ T-cell-dependent acute GvHD was induced in healthy C57Bl/6 female mice by allogeneic hematopoietic stem cell transplantation (aHSCT). Briefly, on the day of aHSCT (aHSCT + Day 0), recipient mice received 11-Gy total body irradiation in two split doses (5.5 Gy each), 3 h apart, using a linear accelerator (1.07 Gy/min). Irradiated animals were then transplanted with $3x10^6$ allogeneic bone-marrow cells and 1.5 $9x10^7$ splenocytes via retroorbital injection, under ketamine-xylazine anaesthesia. In most experiments, aGvHD was induced using OT-I mice as donors and Act-mOVA mice as recipients (OT-I → ActmOVA model), both on the C57Bl/6 background. After aHSCT, mice were monitored daily for weight loss, hunchback, ruffled fur, apathy, and (unless otherwise stated) euthanized on aHSCT + Day 4 by CO2 asphyxiation. For control experiments, recipient mice were also grafted with CD8+ T-cell-depleted grafts (OT-I/CD8 depleted → Act-mOVA), CD8+ T-cell-depleted grafts spiked with increasing numbers of OT-I T cells (OT-I → ActmOVA), syngeneic grafts (Act-mOVA → Act-mOVA), or grafts with multiple minor, and major histocompatibility antigen disparities (Balb/c → B6 aGvHD system)

Histology

For the histological analysis, mice were sacrificed at the day 0 and day 4 and the skin, small intestine, lungs and liver were removed. Next, 5-lm thick FFPE sections were prepared, and standard haematoxylin and eosin staining were performed. Images were captured with an inverted microscope using a 10X objective in transmitted light mode. Tissue histology was evaluated by two independent pathologists.

ELISA

For the ELISA analysis, CD45.1/OT-I CD8+ T cells were collected from the grafts on aHSCT + Day 0, before grafting, to serve as a reference. Next, on aHSCT + Day 4, grafted CD45.1/OT-I CD8+ T cells were also retrieved from the peripheral blood, small intestine, lungs, and liver of Act-mOVA mice, displaying acute GVHD. Cells were plated on 96 well plates at a density of 10^4–10^5 cells/well, and cultured in RPMI-Glutamax supplemented by 10 % FBS for 48 h at +37ºC in a 5 % CO2 atmosphere. Culture supernatants were analyzed by a Mouse Granzyme-B ELISA kit, following the manufacturer's instructions. Granzyme-B release was assessed using a Multiskan MS ELISA reader and normalized to cell numbers.

TREC assay

For TREC assays, CD45.1/OT-I CD8? T cells were sorted from the graft on aHSCT + Day 0 as controls. In addition, grafted CD45.1/OT-I CD8+ T cells were also retrieved from the peripheral blood, small intestine, lung, and liver of transplanted Act-mOVA mice affected by acute GVHD on aHSCT + Day 4, as well. Genomic DNA was isolated from all OT-I T cells using NucleoSpin Blood kits. Next, copy numbers of T-cell receptor excision circles (TRECs) were assessed by Q-PCR. A standard reference copy number assay was used as an internal reference. Amplifications were done using the Platinum Quantitative PCR Super Mix kits, a 7900 HT Fast RT PCR system and the following PCR protocol: +95 ºC for 10 min and then +95 ºC 15 s, +95 ºC 1 min for 45 cycles. The results were interpreted using the ddCt method.

Flow cytometry

Flow cytometry was performed using PE-Cy7-CD8b, FITC-CD45.1, FITC-granzyme-B, FITC-IFN gamma antibodies. Cell viability was assessed with help of the LIVE/DEAD Fixable Dead Cell Stain Kit. For intracellular staining, cell permeabilization was carried out using the Cytofix/Cytopermkit. Data were collected on a FACS Calibur flow cytometer and analysed by FlowJo.

Gene expression profiling

Total RNA was extracted from CD45.1/OT-I CD8+ T cells using the RNeasy Plus Micro Kit. Next, 300 pg of total RNA per sample was reverse transcribed, amplified in two rounds, and Cy3 labeled by the Arcturus RiboAmp PLUS, Cy3 labeling Kit. Amplified samples were hybridized to 4 9 44 k mouse whole-genome microarrays and scanned on an Agilent Microarray Scanner. Raw data were retrieved by the Feature Extraction software, imported into GeneSpring, quantile normalized, and the baseline was set to the median of all samples for every gene analysed. Next, all genes flagged as not detected or not displaying signal intensities higher than the 20th percentile of all detected signals in at least one experimental group were discarded. The remaining gene set was analysed by the principal component analysis (PCA) and further screened to identify differentially expressed genes (DEGs). To this end, any genes displaying [twofold change between CD45.1/OT-I CD8? T cells on aHSCT + Day 0 (graft, serving a control) and any of the aGVHD-affected experimental groups on aHSCT + Day 4 (peripheral blood, small intestine, lungs, and liver) were identified, and evaluated using One-Way ANOVA and the Benjamini– Hochberg multiple testing correction (p\ 0.05). Within this gene set,

Tukey's pairwise analysis was applied (p\ 0.05) to identify DEGs passing selection criteria in at least one of the four possible pairwise comparisons. This gene set was further analysed by the gene set enrichment analysis (GSEA), and select genes were also displayed on heat maps generated by the Interactive Heat map Viewer of the BRB-Array Tools software. Raw microarray data have been deposited at the GEO database (GSE79083).

Methods used in human experiments

Blood samples

Blood samples were collected from selected aHSCT patients of the Department of Haematology and Stem Cell Transplantation, St. Istvan and St Laszlo Hospital, Budapest, Hungary for research purposes, after obtaining informed consent and IRB approval. aHSCT patients belonging to the following groups were recruited:

(i) patients developing no acute GvHD following aHSCT,
(ii) aHSCT patients with acute cutaneous GvHD,
(iii) aHSCT patients with acute GI GvHD, or
(iv) aHSCT patients with both acute and GI aGvHD

Samples from aHSCT patients without acute GvHD were collected after ca. 30 days post aHSCT (no GvHD group). Samples from aHSCT patients with aGvHD were collected at the time of diagnosis, before any GvHD-specific treatment had been commenced (GvHD groups). In addition, control blood samples were also collected from healthy volunteers, as well (n = 10). Blood was collected in ACD-A (Acid Citrate Dextrose) and EDTA (ethylenediamine tetraacetic acid) tubes.

Tissues

Human skin and intestinal biopsies were obtained from patients of the Department of Dermatology, Venereology and Dermatooncology, Budapest, Hungary, and the Uzsoki Hospital of the Semmelweis University, Budapest, Hungary, respectively.

Isolation of peripheral blood mononuclear cells

Samples were stored and transported at room temperature (RT). PBMCs were isolated by density centrifugation using Histopaque-1077, frozen at −80°C and transferred to liquid nitrogen until further use.

FACS sorting

FACS sorting was used to isolate skin- and gut-homing CD8+ T cells for microarray gene expression profiling. Frozen PBMC samples were thawed and immediately stained with LIVE/DEADR Fixable Dead Cell Stain, CD8β-APC, CLA-FITC, and ITGβ7-PE antibodies.

Three-way sorting of PBMCs was done on a BD FACS Aria III sorter:

- Skin-homing Ly/LIVE/DEADR –/CD8β+/CLA+ T cells,
- Gut-homing Ly/LIVE/DEADR –/CD8β+/ ITGβ7+ T cells,
- and Ly/LIVE/DEADR –/CD8β+/CLA–/ITGβ7– T cells, the latter serving as reference,

Viability of sorted T cells was >95% by LIVE/DEAD staining. T cells were sorted directly into RLT Plus lysis buffer and the cell lysates were subsequently frozen at −80°C.

Gene expression profiling

Total RNA was extracted from FACS-sorted CD8+ T cell subsets using the RNeasy Plus Micro Kit. Sample integrity and yield were assessed on a 2100 Bioanalyzer using an RNA 6000 Pico Kit. Three-thousand picograms of total RNA per sample was amplified in two rounds and Cy3-labeled by the Arcturus RiboAmp HS PLUS, Cy3 labelling Kit. Cy3-labeled amplified cRNA was hybridized to human GE 4x44K v2 whole-genome microarrays and scanned on an Agilent Microarray Scanner. Raw data were retrieved with the Feature Extraction software. Analysis were performed using the BRB-Array Tools developed The following genes were excluded from further analyses: genes flagged as not detected, genes displaying batch adjusted signal intensities lower than log2 = 1, genes not detected in all samples of any experimental groups, and genes not displaying twofold change in at least one experimental group in either direction from the given gene's median value. The remaining gene set was analysed by two-way RM ANOVA and Benjamini-Hochberg multiple testing correction (FDR < 0.01) to identify differentially expressed genes. Microarray data have been deposited at GEO under accession number GSE103569.

qPCR

Total RNA was extracted and reverse transcribed from T cell subsets. PI16 gene expression was assessed with human PI16-specific TaqMan probes and HGPRT as internal reference. cDNA was amplified on a 7900HT Fast Real-Time PCR machine using 2× Sensifast Probe HI-ROX master mix. Results were calculated using the comparative CT (CT) method.

Flow cytometry

For flow cytometry, cells were stained with CLA-FITC, IFN-γ-FITC, integrinβ7-PE, PI16-PE, CCR10–PE, CCR4–PE, CCR8-PE, CD8β–APC, CD8β-PECy7, CD3-APC, CD59-FITC, CD14 PerCPCy5.5, CD56-APC, granzyme-B–FITC, CD69–FITC, CD127–FITC, CD25–PerCP-eFluor710, CD45RO PerCP-eFluor710, PI16-APC antibodies and appropriate isotype controls following standard protocols. Intracellular antigens were stained using Brefeldin-A and Fixation/Permeabilization Solution. Cells were analyzed on a FACS Calibur flow cytometer. Data were evaluated using the FlowJo v10.1 software.

Immunocytochemistry

CD8β+ T cells were blocked with mouse and rat sera and stained with CLA–FITC, PI16–PE, and CD8β-APC antibodies. For APC detection, signal amplification was done using anti-APC-biotin and Streptavidin APC. After staining, cytospin slides were prepared, and samples were covered with Fluoroshield, with DAPI histology mounting media overnight, at RT. Images were taken on an FV500 inverted laser scanning confocal microscope at 60× magnification. Image analysis was performed with the Image J v. 1.8.0 112 software.

Immunohistochemistry

FFPE tissue sections (5 μm) were prepared from healthy human skin biopsies. Antigen retrieval was done in sodium citrate buffer (pH 6.0) at +105°C for 30 min. Samples were blocked with 10% FBS and stained with rat anti-human CLA-FITC (1:10), mouse anti human CD8a and rabbit anti human PI16 (1:100) antibodies. Secondary antibodies used were anti-mouse IgG-eFluor570 (1:100) and anti-rabbit IgG-APC

(1:100). Staining controls were done by omitting primary antibodies. Images were captured with an inverted confocal microscope at 20× magnification and evaluated with Image J v. 1.8.0 112.

GPI anchor digestion

CD8β+ T cells were washed twice with PBS, and 1 × 106 cells/500 μL were left untreated or exposed to various doses of Bacillus cereus PI-PLC, as indicated, for 1 h at RT. Cells were immediately stained and analysed by flow cytometry.

RA and 1,25-dihydroxivitamin D3 treatment

Sorted CD8β+ T cells were seeded on 24-well plates at a density of 7 × 105 cells/well, in glutamine-supplemented RPMI 1640, 10% human serum type AB, 1× Glutamax, 1% Penicillin/streptomycin. Culture media and sera were pretested for assay performance in pilot studies. Cells were activated with Dynabeads CD3/CD28 at a cell:bead ratio of 1:3 for 2 days in the presence of 2.5 ng/mL recombinant human IL-12. After 2 days, cultures were supplemented with recombinant 12.5 ng/mL IL-2, split into three and treated with 10−8 M RA, 10−8 M 1,25-dihydroxivitamin D3 (D3), or vehicle only (0.1% ethanol). Every 2 days till day 10 post activation, cultures were split into two, supplemented with fresh chemicals and media, and analysed by flow cytometry

Pull-down assay and proteome profiling

Human skin samples, obtained as above, were immediately transferred to a ProteoJet Mammalian Cell Lysis Reagent, supplemented with HaltTM Protease Inhibitors Cocktail and preincubated at +4°C for 30 min. Next, subcutaneous fat was

removed and samples were cut in 1–3 mm pieces by sterile scalpels and homogenized with a Diax 100 homogenizer. Debris were removed by centrifugation. Skin lysates were stored at −80°C until further use. For protein pull down, 10 μg recombinant GST-tagged human PI16 was dialyzed using Slide-A-LyzerTM Dialysis Cassettes. After removal of excess glutathione, PI16 was bound to glutathione-linked agarose beads of the PierceTM GST Protein Interaction Pull-Down Kit. Pull-down assay was performed with 100-μg skin protein lysate, following the manufacturer's instructions. Proteome profiling was used to compare the following fractions in a pair-wise manner:

(i) specific pull-down fraction (PI16-bound agarose beads),
(ii) aspecific binding control (empty agarose beads),
(iii) whole skin lysate before pull down,
(iv) flow-through fraction after pull down.

Skin proteases specifically bound to PI16 in pull-down assays were identified using Proteome ProfilerTM Human Protease Array Kits. PI16-bound skin proteases were identified by comparing the specific pull-down fraction with the aspecific binding control, and the whole skin lysate with the flow-through, respectively. Sample preparation, membrane incubation, and signal detection were all carried out following the manufacturer's instructions. Densitometry was done using the FluorChem Alphaview software.

4. Results

Development of the OT-I → OVA acute GvHD model

In the first part of my thesis, we managed to set up and successfully validate a new, TCR-modified, single miHA mismatch-based mouse experimental aGvHD model.

We described a robust experimental model of aGvHD based on two commercially available transgenic mouse strains, capable of inducing highly reproducible, lethal aGvHD, to our best knowledge in 100 % of the recipient animals, rapidly, within approximately 4–7 days.

Acute GvHD was induced by lethal dose TBI and subsequent aHSCT involving two transgenic, single minor allele mismatched, but otherwise syngeneic mouse strains as aHSCT donors and recipients. To this end, C57Bl/6 ActmOVA mice carrying an ubiquitously expressed membrane-bound chicken ovalbumin (OVA) transgene under the control of the CMV immediate-early enhancer and chicken beta-actin promoter, and displaying the OVA254–267 (SIINFEKL) peptide in the context of H2-Kb, were used as recipients; while C57Bl/6 OT-I animals, featuring CD8+ T cells with a transgenic TCR restricted to H2-Kb-presented SIINFEKL peptides, were utilized as donors. The model recapitulates some clinical aHSCT conditions that its alternatives usually do not, and reproduces the pathology of aGvHD occurring in human patients, too. Nevertheless, unexpectedly the skin showed no CD8+ T cell infiltration, CD8+ T cell clonal expansion, or tissue damage at all. Microarray analysis showed that the model might be sensitive enough to

provide insight into tissue-specific differences in the functional properties of CD8+ T cells during aGvHD, as well. Apparently, in the small intestine, a large gene cluster of IL2R subunits and several cyclins both closely linked with each other and associated with rapid clonal expansion following T-cell activation, are less intensively transcribed than in any other target organ of aGvHD. However, due to the lack of aGvHD expression in the skin, the skin-gut specific homing comparison of CD8 + T cells has not been performed and our model is a system complementary to a known, single miHA mismatch-based aGvHD model (K14-OVA).

Analysis of CD8 + T-cell homing markers on human samples

In the second part of my thesis, I performed a hypothesis-free investigation of human aGvHD samples in order to identify differentially expressed in distinct homing subsets of CD8+ T cells. This dissertation presents the validation and functional analysis of only one gene (PI16) that is not yet known in this context. We found that PI16 is over-expressed on non-naïve, resting memory-like CLA+/CD8+/CD45RO+ T cells homing to the skin, and confirmed that it is a novel, disease-independent skin-homing biomarker of this cytotoxic T cell population. This finding is important from a basic research point of view, but its clinical value is probably small, as it is not related to any aGvHD manifestations. By performing confocal microscopy on sorted CD8+ T

cells, we found that human skin homing CLA+ T cells express a membrane bound, GPI-anchored form of PI16.

Regarding the regulation of PI16, we found that induction of PI16 on skin-homing CD8+ T cells seems to be independent of known factors inducing other skin-homing CD8+ T cell biomarkers, and particularly those affecting CLA expression. On the other hand, we have clearly demonstrated that the persistence of PI16 on resting CD8+ T cells continues until these cells reactivate and reach the effector phenotype. Stimulation of peripheral PI16+ T cells resulted in rapid downregulation of this marker. Regarding the function of PI16, the tests we carried out have supported the results of mouse experiments that human PI16 on skin-homing T cells may act as a partial inhibitor of cathepsin K in the human skin, but may not bind to other skin proteases with high affinity. All this offers many interesting speculations on the function of the CD8 + T cells in the skin such as whether T-cells activated in inflammatory responses can inhibit the inhibition of the proteases necessary for the inflammatory process, i.e., can affect the remodelling of the local matrix. However, to clarify this issue more clearly first we need to understand the mysterious and virtually unknown CAP domain of PI16, which maybe help us to understand deeper the function of PI16.

5. Conclusions

Studies on the OT-I → OVA acute GvHD model

1. In the first part of my thesis, we managed to set up and successfully validate a novel TCR-transgenic murine aGvHD model developing aGvHD due to a single, defined miHA antigen mismatch.
2. The model induced aGvHD in the recipient animals with 100% lethality in highly reproducible manner, within 4-5 days, affecting a wide variety of typical target organs, surprisingly however, it failed to develop cutaneous aGvHD.
3. This unexpected observation be explained by the fact that while other organs developed aGvHD 4 or 5 days after transplantation, the skin would have taken much longer time because of the small number of available OVA-presenting APCs. In other words, it is possible that the Act-mOVA model simply did not have enough time for the appearance of skin symptoms due to dysfunctionality of other aGvHD target organs that were more severely damaged.
4. Our model is a system complementary to a known, single miHA mismatch-based aGvHD model (K14-OVA) that, however, can develop cutaneous aGvHD only. In contrast to the K14-OVA system, our model delivers a rapid lethal gastrointestinal / hepatic / pulmonary aGvHD with the exception of the skin involvement.
5. The model provides an opportunity for tissue-specific tracking, recovery and phenotyping of CD8 + T cells homing to, or establishing residence in the target organs during aGvHD, thus enabling a deeper

understanding of the mechanism of the disease. Furthermore, with some modifications, our system is suitable to investigate cross-presentation or even GvL effect as well.

Study of CD8 + T-cell homing markers on human samples

1. We described peptidase inhibitor 16 (PI16) as a novel marker of cutaneous T cell homing, as we demonstrated that it was preferentially expressed by skin-homing T cells. PI16 expression was restricted to the non-naïve, resting CD8+/CLA+/CD45RO+ T cell subset.
2. Clinical value of PI16 as a marker of aGvHD or cutaneous aGvHD is believed to be limited, however, as it is not related to any of the aGvHD organ manifestations.
3. Hence, in these studies, aGvHD was utilized as a model system allowing parallel analysis and characterization of skin and gut homing CD8+ T cells, but we were unable to identify any T cell homing markers related to the disease itself or its different organ manifestations.
4. PI16 is exclusive to T cells and it binds to their plasma membrane through a GPI anchor.
5. Expression of PI16 by skin-homing CD8+T cells is maintained even after their extravasation to the skin, at least in a healthy skin remaining under steady state conditions is still.
6. Reactivation of skin homing CD8+ T cells, however, results in rapid loss of PI16 induced by transcriptional down-regulation.

7. Activation of PI16 expression on skin-homing CD8 + T cells occurred independently of known factors responsible for the imprinting of a skin homing T cell phenotype, and particular those influencing the expression of CLA.
8. PI16 functions as a partial inhibitor of cathepsin K, a skin protease relevant to inflammatory skin conditions.

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