### Title

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### Journal

International Journal of Women's Dermatology, 6(4)

### ISSN

2352-6475



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### Publication Date

2020-09-01

### DOI

10.1016/j.ijwd.2020.05.009

Peer reviewed

International Journal of Women's Dermatology 6 (2020) 311–317

Contents lists available at ScienceDirect

International Journal of Women's Dermatology

Original Research

# Local immune cell infiltration in cutaneous acute graft versus host disease

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ARTICLE INFO

*Article history:*
Received 2 April 2020
Accepted 15 May 2020

*Keywords:*
Graft versus host disease
Acute GVHD
Cutaneous GVHD
T-cell lymphocytes
Macrophages

ABSTRACT

*Background:* Hematopoietic stem cell transplant is a crucial intervention to definitively treat many hematopoietic malignancies, but it carries great risks of morbidity and mortality often associated with graft-versus-host disease (GVHD). Acute and chronic GVHD are distinct entities, defined by a combination of historical, clinical, and pathologic data, but both are generally thought to stem from self-propagating aberrantly activated immune cells inflicting end organ damage, with the potential to cause significant illness or even death. Event-free survival rates after hematopoietic stem cell transplant continue to improve each year, but GVHD remains a major hurdle in improving the efficacy and safety of transplant.

*Objective:* Recent studies demonstrating tissue-specific immune effector phenotypes underscore the need for a deeper understanding of the cellular and molecular pathways driving the destruction of target tissues in patients with acute GVHD.

*Methods:* Samples were collected from lesional and unaffected skin in five patients with acute cutaneous GHVD. Fresh tissue was processed for fluorescence-activated cell sorting and analysis of macrophages and lymphocytes.

*Results:* The percentage of lymphocytes and macrophages as a representation of total cells varied among patients and was not always consistent between lesional and unaffected sites. The heterogeneity in immune cell profiling observed in patients in this study could reflect the diverse demographics, conditioning, and transplant conditions of each individual.

*Conclusion:* This study provides initial insight into the underlying molecular mechanisms of cutaneous GVHD progression and paves the way for additional studies to examine the cellular and molecular landscape in greater detail.



## Introduction

Hematopoetic stem cell transplantation (HSCT) is used as a curative therapy for hematologic malignancies, but its therapeutic potential is limited by a number of complications, including infection, graft failure, and graft-versus-host disease (GVHD). GVHD reportedly occurs in at least 40% to 60% of transplanted patients and accounts for 15% of the mortality seen after HSCT (Strong Rodrigues et al., 2018). The severity of acute GVHD is directly related to the degree of mismatch in human leukocyte antigen (HLA), the genes encoding major histocompatibility complex proteins in humans. Additional important factors include minor histocompatibility antigens, age, myeloablative conditioning regimen, sex disparity, cytomegalovirus serology status, donor multiparity, and the use of peripheral blood stem cells (Strong Rodrigues et al., 2018). Outcomes are improved when donors and recipients are matched at high resolution with at least four HLA loci. However, although HLA matching reduces the risk of GVHD, 40% of patients receiving HLA-identical grafts still develop acute GVHD due to differences in minor histocompatibility antigens that lie outside the HLA loci (Goulmy et al., 1996).

Because the skin is often the first and most commonly affected organ in GVHD, dermatologists play a crucial role in the diagnosis and management of these patients. The cutaneous eruption of acute GVHD can present on a spectrum of severity, ranging from

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https://doi.org/10.1016/j.ijwd.2020.05.009
2352-6475/

macular erythema to severe forms mimicking Stevens–Johnson syndrome or toxic epidermal necrolysis (Kavand et al., 2017). The rash usually starts within 1–3 weeks after engraftment and often appears as a maculopapular eruption that can be painful, itchy, or asymptomatic (Kavand et al., 2017). The rash typically starts on the neck, face, and acral extremities and can progress to affect the entire body. Gut or liver involvement is also typical of GVHD and may be heralded by elevated bilirubin levels, diarrhea, persistent nausea, and/or abdominal pain. The disease is classified into different stages depending on the severity of end organ involvement.

Current work attempting to understand GVHD on a more cellular level outlines three overarching stages. Initially, tissue injury incurred during the conditioning regimen promotes an exaggerated inflammatory response (Nassereddine et al., 2017). This is followed by donor T cell activation by recognition of alloantigens. Next, activated T cells release cytokines to propagate their own expansion and promote ongoing activity, in addition to aberrantly activating other immune cells. This self-perpetuating cycle ultimately leads to tissue destruction and end organ damage disproportionally affecting the skin, liver, and gut.

Skin biopsy and histopathologic analysis can contribute to the diagnosis of GVHD, but newer studies have focused on identifying biomarker panels to predict the development and severity of acute GVHD with greater accuracy (Budde et al., 2017; Paczesny et al., 2009a,b). Part of elucidating these biomarkers involves understanding what types of immune cells and cytokines are present in the skin and blood of these patients and how they might be interacting. Prior studies have looked at mRNA expression of certain genes in skin biopsies of acute GVHD and found an upregulation of interleukin (IL) 2, IL-4, and interferon-gamma (Roy et al., 1995). Other investigators have found interferon-gamma and IL-17 to be abundant in murine acute GVHD of the skin, but no predominate cytokine was expressed in human skin samples (Lai et al., 2012). One study looked at the cellular profile in lesions of acute GVHD and found T cells to predominate, whereas $CD191^{+}$ B cells, natural killer cells, and granulocytes were almost absent (Roy et al., 1995).

In summary, although attempts to understand the precise underpinnings of this process have identified several upregulated cytokines and related signaling cascades, a thorough understanding of the extent of immune cell dysregulation in acute GVHD is still lacking. Furthermore, there is a specific paucity of data regarding the site-specific changes that occur within affected tissues, such as the skin. The current study aimed to characterize the local immune milieu promoting cutaneous disease by comparing the presence of lymphocytes and macrophages in matched lesional and unaffected skin samples from patients with acute GHVD. A better understanding of the molecular and cellular pathways driving the initiation and progression of cutaneous GVHD would help identify early cases and potentially even prevent some of the significant morbidity associated with this diagnosis.

## Methods

### *Patient recruitment and sample collection*

Patients presenting to the dermatology clinic or those evaluated by the inpatient dermatology service between December 2018 and June 2019 were recruited to participate in the study. The inclusion criteria included HSCT patients aged $\geq$18 years at the time of consent with a rash covering at least 5% body surface area (BSA) and suspected of having acute cutaneous GVHD. The main exclusion criteria were a lack of unequivocal histopathologic findings supporting a diagnosis of acute GVHD or clinical progression of symptoms that favored an alternate diagnosis.

After obtaining informed consent, three 4-mm punch biopsies were collected from each patient, two from lesional and one from unaffected skin. In one case, a shave biopsy was taken from lesional skin. One of the lesional skin specimens was placed in formalin for routine hematoxylin and eosin processing and diagnostic reading by a dermatopathologist (B.H.) to confirm the clinical suspicion of acute GVHD. The two research samples (one lesional and one control/unaffected) were stored on saline-soaked gauze until further processing, as detailed later. Representative sites were chosen by the clinical appearance of rash (e.g. presence of erythema, edema, papules on lesional skin) or lack thereof in the case of the control specimens.

### *Tissue digestion and cell counting*

Skin biopsy samples were processed within 4 hours of sample collection in an overnight digestion in dissociation media (RPMI 1640 with 10% FCS, 1 mg/mL collagenase, 2 mM L-glu, penicillin 100 U/mL, and streptomycin 100 μg/mL) at 37 °C to create a single cell suspension. The next morning, DNAse was added to a final concentration of 200 U/mL, and the sample was incubated at room temperature for 15 min. Samples were mechanically disrupted and then iteratively washed through a series of filters (100 μm, 70 μm) using ice cold HBSS with EDTA. After each wash, samples were centrifuged for 5 min at 1200 RPM. After the final round of washing, cells were counted using trypan blue and a manual hemocytometer. Finally, cells were resuspended in 70% DMSO with FCS for storage at −80 °C while awaiting further analysis.

### *Fluorescence-activated cell sorting and analysis*

Once five patient samples from lesional and unaffected skin were collected, the samples were thawed, washed, and resuspended prior to fluorescence-activated cell sorting (FACS) analysis. Antibodies were used to identify macrophages (CD14, HCD14), lymphocytes (CD3, HIT3a), and lymphocyte subtypes (CD4 OKT4, CD8 HIT8a; Biolegend, San Diego, CA). The samples were analyzed using BDFACSCanto or LSR II instruments with FACSDiva software, and data were analyzed using FlowJo, version 10, along with Prism software (BD Biosciences, San Jose, CA). Statistical analysis was performed in Prism using the Wilcoxon matched pairs signed rank test.

## Results

### *Patient characteristics and outcomes*

Six patients met the inclusion criteria for the study and were enrolled. One patient went on to develop upper respiratory symptoms and an uptrending adenovirus titer and was therefore excluded from the study based on clinical suspicion for a concomitant viral exanthem. The baseline characteristics of the five patients included in the study are outlined in Table 1. Patient age ranged from 45 to 71 years, with a mean age of 59 ± 0.8 years. Patients had diverse indications for HSCT. Four of five patients had matched unrelated donors, and the fifth patient had an HLA-matched sibling donor. Three transplants were sourced from peripheral blood, and the other two were sourced from bone marrow.

The onset of cutaneous GVHD occurred prior to post-transplant day 100 in all five cases, and all patients presented with some variation of pink macules and/or papules scattered over the extremities and trunk. Representative images are shown for all patients in Fig. 1, some of which highlight the specific sites from which samples were collected for additional study. The median time after

**Table 1**
Clinical characteristics of all five patients.

| Patient | Age (y), sex | Diagnosis | Type of preconditioning regimen | Date of transplant | Type of transplant | Stem cell source | Date onset rash | Biopsy sites |
|---|---|---|---|---|---|---|---|---|
| 1 | 59, F | DLBL; Richter's syndrome | Fludarabine and busulfan | 12/11/18 | Matched unrelated donor; allogeneic SCT | Bone marrow | Day 27 | Left back; right flank |
| 2 | 45, M | Plasma cell leukemia | Cytoxan/total body irradiation | 9/18/18 | Matched unrelated donor; allogeneic SCT | Peripheral blood | Day 98 | Right leg; right chest |
| 3 | 52, F | DLBL | Thiotepa-fludarabine-cyclophosphamide; Rituxan | 2/11/19 | Sibling allogeneic SCT (after relapse of autologous) | Peripheral blood | Day 29 | Right hand; right forearm |
| 4 | 71, M | Accelerated phase CML | Fludarabine and busulfan | 1/26/19 | Matched unrelated donor; allogeneic SCT | Bone marrow | Day 47 | Left abdomen; right leg |
| 5 | 68, M | Accelerated phase CML | Reduced intensity conditioning with fludarabine and melphalan | 4/26/19 | Matched unrelated donor; allogeneic SCT | Peripheral blood | Day 35 | Right back; left hand |

| Patient (cont'd) | Pathology grade | Morphology | Distribution of rash | Donor characteristics | Recipient characteristics | Clinical stage of GVHD: Skin | Clinical stage of GVHD: Gut |
|---|---|---|---|---|---|---|---|
| 1 | Lerner grade 2 | Maculopapular | Trunk, face | Male<br>Blood type: O+<br>CMV positive<br>HLA 10/10 match | Female<br>Blood type: A +<br>CMV negative | 3 | 0 |
| 2 | Lerner grade 1 | Mostly macular | Trunk, proximal extremities | Blood type: O+<br>CMV negative<br>8/8 (with a single DQ mismatch) | Blood type: A +<br>CMV negative | 2 | 0 |
| 3 | Lerner grade 2 | Papular on dorsal hands<br>Mostly macular erythema<br>25%-50% BSA | Neck, back with macular erythema; palms, soles with erythema and scale; firm papules on dorsal hands | Male sibling<br>Blood type: A+<br>CMV negative | Female<br>Blood type: A +<br>CMV negative | 2 | 0 |
| 4 | Lerner grade 2<br>*Lichenoid | Maculopapular | Trunk, upper extremities, dorsal hands, proximal lower extremities | Male<br>Blood type: B +D+<br>CMV negative<br>10/10 HLA match | Male<br>Blood type: B +D+<br>CMV positive | 2 | 0 |
| 5 | Lerner grade 2 | Mostly macular with follicular prominence<br>BSA >80% | Trunk, proximal extremities, buttocks; spares distal extremities | Blood type: O+<br>CMV negative<br>10/10 HLA match | Blood type: O +<br>CMV negative | 2 | 0 |

| Patient (cont'd) | Clinical stage of GVHD: liver | Overall GVHD grade | Treatment | Clinical course |
|---|---|---|---|---|
| 1 | 0 | 1 | Fluocinonide ointment; oral prednisone | Resolved over 3 months, then tapered off oral prednisone; progressed to have mild chronic GVHD of the liver |
| 2 | 0 | 1 | Fluocinonide ointment; oral prednisone | Resolved over 4 months, tapered off prednisone |
| 3 | 0 | 1 | Triamcinolone cream; oral prednisone | Improved over 2–3 months, was able to taper to physiologic doses of prednisone |
| 4 | 0 | 1 | Clobetasol ointment; oral prednisone | Deceased |
| 5 | 0 | 1 | Clobetasol cream (Had bad reaction to oral prednisone in past so did not use) | Resolved over 3 months |

BSA, body surface area; CML, chronic myelogenous leukemia; CMV, cytomegalovirus; DLBL, diffuse large B-cell lymphoma; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; SCT, stem cell transplant.
Overall grade of acute GVHD at diagnosis: 0 (none); I (rash on ≤50% of skin, no liver or gut involvement; II (rash on >50% of skin, bilirubin 2–3 mg/dL, diarrhea 500–1000 mL/day or persistent nausea); III (Bilirubin 3–15 mg/dL, or gut stage 2–4, diarrhea >1000 mL/day or severe abdominal pain with or without ileus); and IV (generalized erythroderma with bullous formation, or bilirubin >15 mg/dL).
Clinical stage of GVHD (skin): Stage 0 (no rash, or no rash attributable to acute GVHD); Stage 1 (maculopapular rash, <25% of BSA); Stage 2 (maculopapular rash, 25–50% of BSA); Stage 3 (generalized erythroderma, >50% of BSA); and Stage 4 (generalized erythroderma with bullae formation and/or desquamation).

transplant to onset of rash was 35 days. Four of five patients displayed clinical stage 2 (maculopapular rash involving 25%–50% BSA) cutaneous GVHD, as defined by the Glucksberg grading system, which places an emphasis on the degree of BSA involved. The fifth patient demonstrated slightly more widespread disease, with >80% BSA involved, which indicated stage 3 GVHD of the skin.

On pathology, four of five samples were classified as Lerner grade II GVHD, which corresponds to diffuse vacuolization of basal cells with scattered dyskeratotic bodies. The fifth sample had slightly less epidermal involvement, with only mild vacuolization of epithelial cells noted. Representative histopathology for each patient is shown in Fig. 2. No patients had intestine or liver involvement initially; overall, all five patients had grade 1 GVHD at the time of skin biopsy.

All patients were treated with topical and systemic steroids, except for one who declined oral prednisone due to a history of poor tolerance. Four of five patients slowly improved over the subsequent 3 to 4 months and were able to taper down or off systemic steroids. One patient died during the course of this study due to relapse and progression of disease.

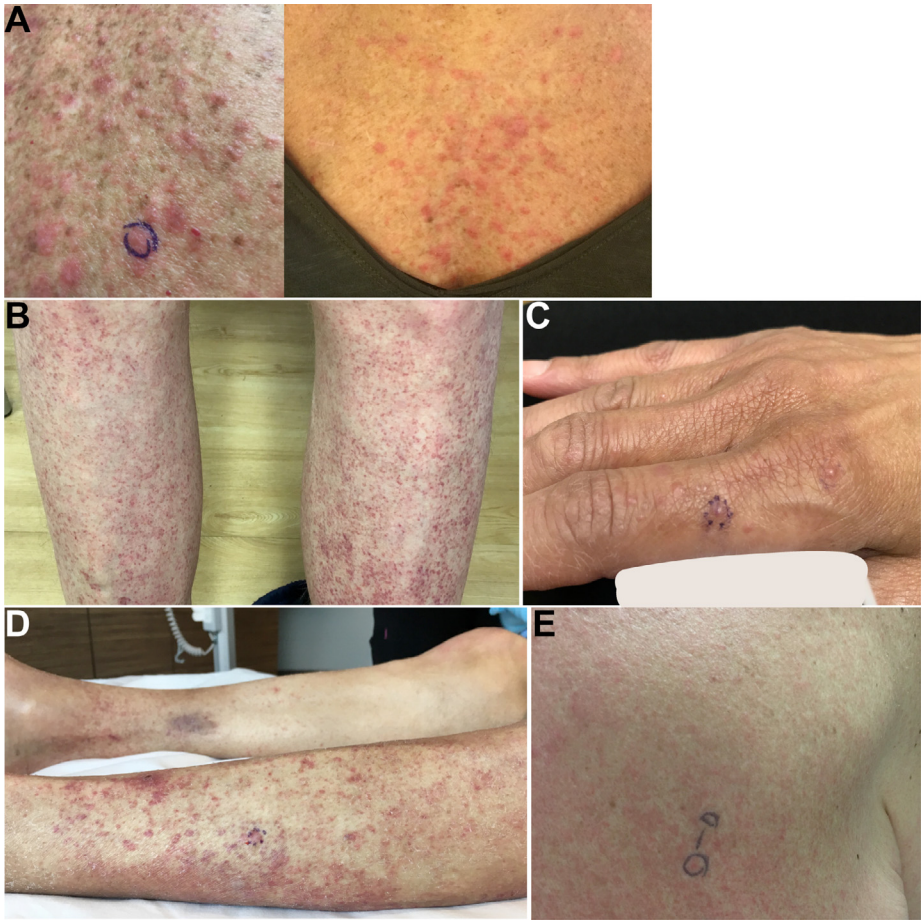


**Fig. 1.** Representative clinical images from all five patients. (A) Patient 1, (B) Patient 2, (C) Patient 3, (D) Patient 4, and (E) Patient 5. A, C, D, and E have the lesional biopsy sites marked.

*Characterization of immune cells in acute cutaneous GVHD*

The total number of cells initially harvested after tissue processing is displayed in Table 2, with counts ranging from $0.70 \times 10^6$ to $4.08 \times 10^6$ total cells from each primary sample. Viability dye confirmed that >68% of cells for all samples were still alive after storage. FACS analysis revealed the heterogeneous nature of each patient's presentation. The percent of $CD3^+$ lymphocytes was increased in lesional skin compared with unaffected skin in three of five cases (Fig. 3A). In four of five cases, the ratio of $CD4^+$ to $CD8^+$ lymphocytes increased in lesional compared with unaffected skin (Fig. 3B). In contrast, $CD14^+$ macrophages were less abundant in lesional compared with unaffected skin in three of five cases, but overall composed only a fraction of immune cells in the analyzed tissue, with representation ranging from 0.2% to 5.9% of all live cells (Fig. 3C).

## Discussion

To better characterize the dysregulated and damaging inflammatory infiltrates in acute cutaneous GVHD, this investigation collected samples from affected lesional skin and clinically unaffected skin from five patients. Primary tissue was processed for FACS analysis with well-established cell surface markers for $CD14^+$ macrophages, $CD3^+$ lymphocytes, and CD4/CD8 T cell subtypes, with the goal to explore changes in the immune cell landscape between clinically involved and spared skin.

Overall, the immune cell presence in lesional versus unaffected skin varied among all five patients, and although the median number of lymphocytes as a percentage of live cells did increase from unaffected to lesional skin, this was not a statistically significant change. There also appeared to be a modest shift in lymphocyte subtype prevalence, with an increased $CD4^+$:$CD8^+$ T cell ratio in lesional versus unaffected skin. Prior studies have highlighted an increase in donor $CD4^+$ T cells in cutaneous GVHD, at least in animal models. Of note, the host versus donor origin of these lymphocytes could not be determined with the current study design (Boieri et al., 2017). Interestingly, two patients appeared to have a greater percentage of $CD3^+$ lymphocytes in clinically unaffected skin compared with a corresponding lesional sample. The sites suspected to be uninvolved in those two patients might have had subclinical activity that evolved to a demonstrable dermatitis in the days after biopsy. On the other hand, this observation might underscore the idea that GVHD is a systemic reaction, with overactivated immune cells present throughout the blood, skin, and other tissues. Additional studies to probe the transcriptional activity and clonal expansion of specific cells at unique lesional sites could be more revealing than an analysis of surface markers alone.

Three of five patients were found to have fewer $CD14^+$ macrophages in lesional compared with unaffected skin, although prevalence as a percentage of live cells varied widely among patients. Monocytes and monocyte-derived macrophages are thought to contribute to GVHD, with evidence suggesting both proinflammatory and protective roles (Ito and Fujino, 2019; Santos e Sousa et al., 2018). In the skin, macrophage infiltration has been associ-

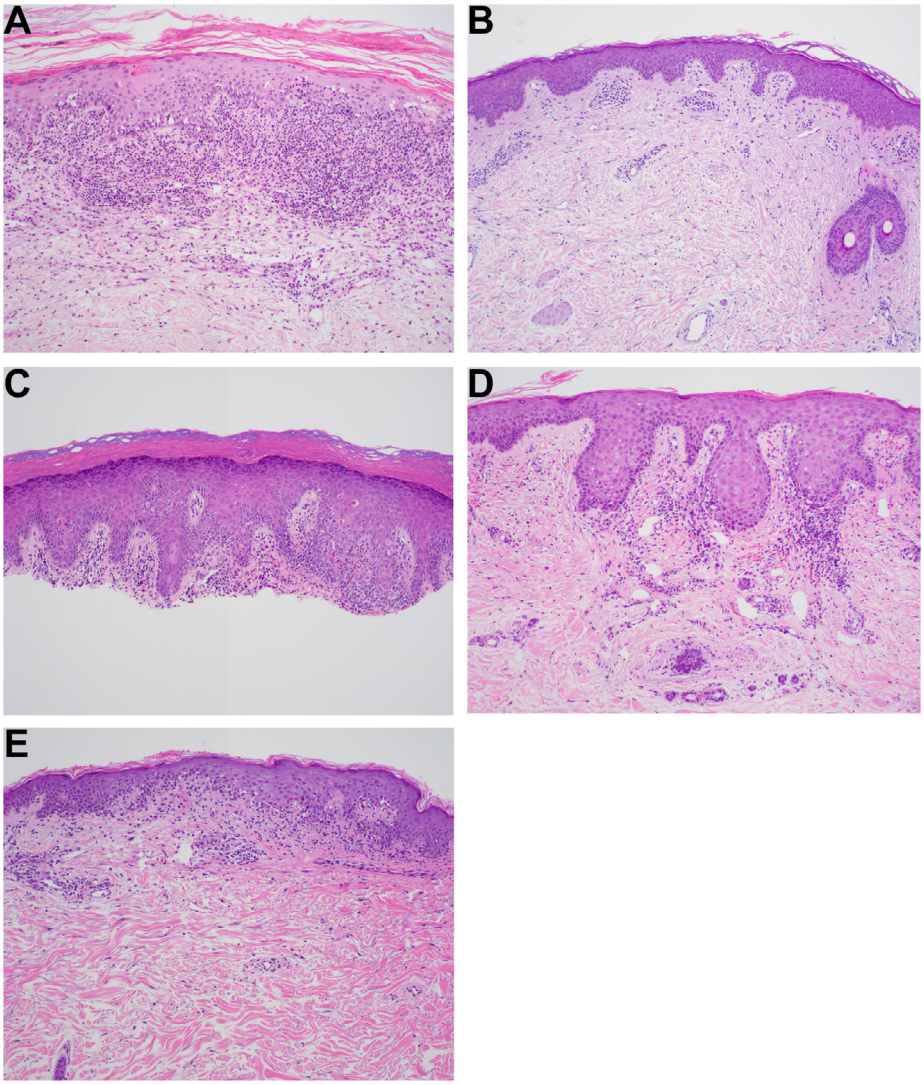


**Fig. 2.** Representative histopathology results at 10× magnification from all five patients. (A) Patient 1, (B) Patient 2, (C) Patient 3, (D) Patient 4, and (E) Patient 5.

**Table 2**
Total cells harvested after initial sample collection and tissue digestion.

| Sample | Cell counts |
|---|---|
| 1 Unaffected | $1.48 \times 10^6$ |
| 1 Lesional | $1.71 \times 10^6$ |
| 2 Unaffected | $4.08 \times 10^6$ |
| 2 Lesional | $1.38 \times 10^6$ |
| 3 Unaffected | $0.73 \times 10^6$ |
| 3 Lesional | $0.70 \times 10^6$ |
| 4 Unaffected | $1.30 \times 10^6$ |
| 4 Lesional | $2.40 \times 10^6$ |
| 5 Unaffected | $1.27 \times 10^6$ |
| 5 Lesional | $1.10 \times 10^6$ |

ated with steroid unresponsiveness and increased mortality (Nishiwaki et al., 2009; Terakura et al., 2015). A deficiency in classical circulating monocytes was also shown to predict increased mortality (de Molla et al., 2019). In this cohort study, the decreased confluence of $CD14^+$ macrophages in lesional skin could reflect the relatively straightforward course for most patients; most recovered from acute GVHD without significant sequelae. The one individual who did prove to have steroid-refractory GVHD (patient 4) demonstrated fewer $CD3^+$ lymphocytes in lesional skin compared with other individuals, but with a marked shift toward $CD4^+$ predominance and a slight increase in $CD14^+$ macrophages in the lesional skin.

The patients recruited to this pilot study had varied histories and exposures, indications for transplant, conditioning regimens, and donor sources. For any one patient, all of these factors, among others, are considered when planning a transplant because some appear to confer a greater risk of subsequent GVHD for reasons not yet well understood (Strong Rodrigues et al., 2018). All five patients initially presented with different degrees of skin involvement and distinct immune cell profiles, possibly reflecting the heterogeneous nature of their disease and transplant conditions. If different aspects of a patient's history or transplant parameters might predispose to a specific clinical appearance at presentation with consistent molecular or cellular changes as well, these may represent unique targets for intervention.

The limitations of this study include the small sample size and the diversity of cases recruited. Future, more involved studies will be necessary to gather information about specific conditioning regimens, donor characteristics, initial diagnosis, and age to understand whether the molecular and immune cell landscape changes adjust for these factors. In addition, only a few cell surface markers were explored in this initial examination owing to limited

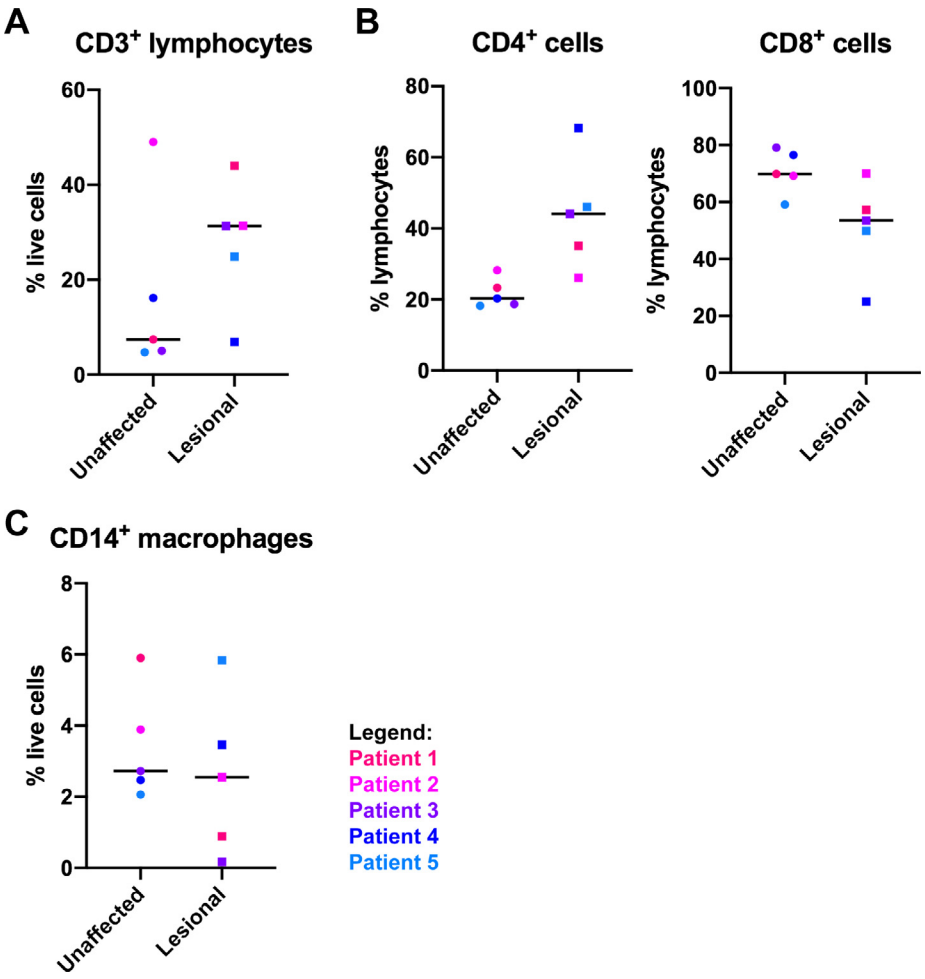


**Fig. 3.** Fluorescence-activated cell sorting analysis of immune cells in unaffected and lesional skin. (A) Dot plot illustrating the percentage of live cells that are $CD3^+$ lymphocytes in unaffected and lesional skin in all five patients sampled. Bar represents median. The difference between the groups was not statistically significant. (B) Dot plots illustrating the distribution of $CD4^+$ and $CD8^+$ cells as a percentage of total lymphocytes. Bars represent median values. (C) Dot plot illustrating the percentage of live cells that are $CD14^+$ macrophages in unaffected and lesional skin in all five patients sampled. Bar represents median value.

tissues available for downstream processing. Future investigations will aim to explore the immune milieu in an unbiased fashion with single-cell sequencing, which will additionally help identify upregulated cytokines and signaling pathways in lesional skin. Ideally, comparison with clinically unaffected skin will lead to deeper insights into the molecular mechanisms of tissue damage in acute cutaneous GVHD.

## Conclusion

In this small pilot study of five patients, an initial investigation into the immune cell types underlying acute cutaneous GVHD revealed the variable presence of macrophages and T-lymphocytes in both lesional and unaffected skin, possibly reflecting the unique response of each patient to an individualized ablation and transplant regimen. This study emphasizes the importance of developing a patient-centric approach when considering GVHD prophylaxis and treatment in the setting of planned HSCT. Future studies to examine the nature of the cutaneous immune infiltrate in even greater detail, through RNA-sequencing or T-cell receptor clonotyping, will help elucidate the molecular mechanisms underlying lymphocyte activation and targeting, potentially revealing additional biomarkers to aid in diagnosis or therapeutic targets to improve patient outcomes.

## Conflict of Interest

None.

## Funding

The Women's Dermatologic Society Academic Research Award.

## Study Approval

The author(s) confirm that any aspect of the work covered in this manuscript that has involved human patients has been conducted with the ethical approval of all relevant bodies.

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