T cell-attracting CCL18 chemokine is a dominant rejection signal during limb transplantation

Graphical abstract

Highlights

- Increased T cell infiltration in the stable graft compared with native skin
- The chemokine CCL18 is upregulated during rejection in limb transplantation
- CCL18 recruits more allo-T cells to the skin and leads to accelerated xenograft loss
- CCR8 blockade halts CCL18-mediated T cell infiltration

Authors

Thiago J. Borges, Phammela Abarzua, Rodrigo B. Gassen, ..., Christine G. Lian, Simon G. Talbot, Leonardo V. Riella

Correspondence

riella@mgh.harvard.edu

In brief

Borges et al. provide a comprehensive immune characterization of limb transplant recipients, demonstrating that the chemokine CCL18 is a dominant signal during rejection. Local CCL18 mediates recruitment of pathogenic allo-T cells into grafts, which is abrogated by CCR8 blockade. Targeting the CCL18:CCR8 pathway is a promising complementary immunosuppressive approach in transplantation.
T cell-attracting CCL18 chemokine is a dominant rejection signal during limb transplantation

Thiago J. Borges,1,2 Phammela Abarzua,3 Rodrigo B. Gassen,2 Branislav Kollar,4,5 Mauricio Lima-Filho,1 Bruno T. Aoyama,1 Diana Gluhova,6 Rachael A. Clark,7 Sabina A. Islam,8 Bohdan Pomahac,7 George F. Murphy,3 Christine G. Lian,3 Simon G. Talbot,4 and Leonardo V. Riella1,2,9,10,*

1Schuster Family Transplantation Research Center, Renal Division, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115, USA
2Center for Transplantation Sciences, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02129, USA
3Program in Dermatopathology, Department of Pathology, Brigham and Women’s Hospital, Boston, MA 02115, USA
4Division of Plastic Surgery, Department of Surgery, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115, USA
5Department of Plastic and Hand Surgery, University of Freiburg Medical Center, University of Freiburg Faculty of Medicine, 79106 Freiburg, Germany
6DF/HCC Specialized Histopathology Core – Massachusetts General Hospital Site, Boston, MA 02129, USA
7Department of Dermatology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115, USA
8Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02129, USA
9Division of Nephrology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA
10Lead contact
*Correspondence: lriella@mgh.harvard.edu
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SUMMARY

Limb transplantation is a life-changing procedure for amputees. However, limb recipients have a 6-fold greater rejection rate than solid organ transplant recipients, related in part to greater immunogenicity of the skin. Here, we report a detailed immunological and molecular characterization of individuals who underwent bilateral limb transplantation at our institution. Circulating Th17 cells are increased in limb transplant recipients over time. Molecular characterization of 770 genes in skin biopsies reveals upregulation of T cell effector immune molecules and chemokines, particularly CCL18. Skin antigen-presenting cells primarily express the chemokine CCL18, which binds to the CCR8 receptor. CCL18 treatment recruits more allo-T cells to the skin xenograft in a humanized skin transplantation model, leading to signs of accelerated graft rejection. Blockade of CCR8 remarkably decreases CCL18-induced allo-T cell infiltration. Our results suggest that targeting the CCL18:CCR8 pathway could be a promising immunosuppressive approach in transplantation.

INTRODUCTION

Vascularized composite allotransplantation (VCA), including limb transplantation, is a life-changing procedure for individuals who have suffered severe traumatic injuries. Unlike solid organ transplantation, VCA involves transplantation of multiple tissues with different immunogenicity levels, including skin, muscle, bones, and nerves. Among these, the skin has the highest immunogenicity of all because of the presence of the following components: a dense population of antigen-presenting cells (APCs) and resident T cells, a rich microbiota, and continuous exposure to environmental threats, both physical and chemical. These unique skin features may explain the 6-fold greater rejection rate of VCA recipients compared with solid organ transplant recipients. More than 85% of individuals undergoing VCA experience acute cellular rejection in the first year after transplantation, and many have multiple episodes of rejection, leading to a higher burden of immunosuppressive therapy over time. It is reported that the number of rejection episodes may portend forthcoming chronic rejection events and graft loss. A better understanding of key distinctions between VCA and solid organ transplantation immune responses is essential for discovery of novel markers of rejection and predictive biomarkers.

Here we identified unique molecular signatures in skin graft biopsies at times of rejection that were dominated by T cell effector immune molecules and chemokines, particularly CCL18. Skin antigen-presenting cells primarily express the chemokine CCL18, which binds to the CCR8 receptor. CCL18 treatment recruits more allo-T cells to the skin xenograft in a humanized skin transplantation model, leading to signs of accelerated graft rejection. Blockade of CCR8 remarkably decreases CCL18-induced allo-T cell infiltration. Our results suggest that targeting the CCL18:CCR8 pathway could be a promising immunosuppressive approach in transplantation.
characterized immunological changes in the graft microenvironment compared with proximal native skin tissue. Our data suggest an increase in T cell infiltration and action in the graft microenvironment compared with native tissue. We demonstrated a role of the chemokine CCL18 in attracting inflammatory allo-T cells to skin grafts, identifying the CCL18-CCR8 pathway as a potential therapeutic target in transplantation.

RESULTS

Expansion of circulating Th17 cells after transplantation
Th17 cells have a pathogenic role in different skin inflammatory disorders.\(^5\,6\) We reasoned that Th17 cells would be enriched over time upon limb transplantation. Three individuals who received limb transplants in our institution between October 2011 and August 2016 were included in this analysis, with a mean follow-up of 5.2 years. The clinical characteristics of these individuals are detailed in Table 1. The individuals’ pre- and post-operative appearance is shown in Figure S1. Detailed information about the individuals’ immunosuppression is given in the STAR methods.\(^5\,6\) We initially characterized T cell subsets from peripheral blood collected over time after transplantation according to our protocol (pre-transplantation, 24 h, 1 week, and 3, 6, and 12 months; Figure 1A). Analyses of the effector and memory T cell subsets (Figure S2) revealed that CD4\(^+\) central memory T cells (TCM cells; CD45RA\(^–\)CCR7\(^+\)) were the predominant T cell phenotype in the pool of CD4\(^+\) T cells, although they decreased after transplantation, whereas CD4\(^+\) effector memory T cells (TEMs; CD45RA\(^–\)CCR7\(^–\)) increased over time (Figure 1B). TEMs and effector memory CD45RA\(^+\) T cells (TEMRA cells; CD45RA\(^+\)CCR7\(^–\)) were the main subsets presented in the pool of CD8\(^+\) T cells with a decrease in naive CD8\(^+\) T cells (CD45RA\(^+\)CCR7\(^+\)) after transplantation (Figure 1C). Next, we assessed the T helper (Th) phenotypes based on CXCR3 and CCR6 expression (Figure 1D). Circulating Th2 cells were the predominant phenotype in individuals with upper extremity transplantation over time (Figure 1E). Th17 cells were increased markedly after transplantation (Figure 1F), whereas Th1 cells were stable over time (Figure 1E). The percentage of circulating regulatory T (Treg) cells (CD4\(^+\)CD25\(^+\)CD127\(^{low}\) cells; Figure 1G) and T follicular helper (Tfh) cells (CD4\(^+\)CXCR5\(^–\)PD-1\(^+\) cells; Figure 1H) had no significant expansion after transplantation, other than transient changes early after transplantation, likely related to use of depletion induction therapy. These findings indicate that Th2 cells were the dominant phenotype over time after transplantation, with a significant expansion of circulating Th17 cells.

Increased T cell infiltration and activation in the non-rejecting graft microenvironment compared with native skin
In transplantation, characterization of the T cells infiltrating human allografts compared with native tissues is technically limited by the small sample sizes collected in punch biopsies. We took advantage of debulking surgeries performed in our limb transplant recipients to examine T cells in the graft microenvironment at nonrejection time points and compared it with recipients’ adjacent native skin removed during the procedure. Allografts and native skins were processed, and infiltrating cells were isolated and stained by flow cytometry (Figure S3). Allografts had higher frequencies of activated CD4\(^+\) T cells, including CD4\(^+\) TEM cells (Figure 2A), Th1 cells (CD4\(^+\)CXCR3\(^+\)CCR6\(^–\)); Figure 2B), and Th17 cells (CD4\(^+\)CXCR3\(^–\)CCR6\(^+\); Figure 2C) compared with native skin. Similarly, total CD8\(^+\) cells (Figure 2D), CD8\(^+\) TEM (Figure 2E) and TEMRA cells (Figure 2F) were also increased markedly compared with native skin. Immunofluorescence

| Recipient characteristics | Individual 1 | Individual 2 | Individual 3 |
|---------------------------|--------------|--------------|--------------|
| Age at transplantation (years) | 65 | 40 | 30 |
| Sex | male | male | male |
| Ethnicity | white | white | white |
| Cause of injury | septic shock | septic shock | ballistic trauma |
| Surgery | bilateral forearm | bilateral upper extremity | bilateral upper extremity |
| PRA (%) | 0 | 69 | 0 |
| Donor-specific antibodies | negative | positive | negative |
| HLA mismatch (A, B, DR) | 5/6 | 5/6 | 4/6 |
| CMV status | negative | negative | negative |
| EBV status | positive | positive | positive |
| Induction agent | thymoglobulin | thymoglobulin | thymoglobulin |
| Follow-up (years) | 9 | 6 | 4 |

| Donor characteristics | | | |
|-----------------------|-----------------|-----------------|-----------------|
| Age (years) | 44 | 23 | 27 |
| Sex | male | male | male |
| CMV status | negative | negative | negative |
| EBV status | positive | positive | positive |
| Total ischemia time (hours) | 4 | 4 | 4 right/5 left |
analyses of skin biopsies confirmed an increased influx of CD4+ and CD8+ cells in the allograft tissue compared with recipients’ adjacent native skin (Figure 2G), with approximately 5–10 positively labeled cells per vascular profile (CD8:CD4 ratio approximately 1:1) in allografts versus only rare ones (1–2 T cells per vascular profile) in adjacent native skin. Our data suggest that the graft microenvironment with alloantigens favors infiltration, expansion, and activation of T cells locally.
Expansion of circulating Th1 and TEM cells during rejection

In our cohort, all individuals developed at least one episode of acute cellular rejection within the first 3 years of transplantation (a total of 9 episodes; range, 2–4); about half occurred during the first 3 months after transplantation, whereas the remaining occurred later (>1 year after transplantation). Clinical aspects of the rejection included a maculopapular rash and edema (Figure 3A). Acute cellular rejection was assessed using Banff grading of skin-containing composite tissue, and most clinical rejection episodes were classified as between grades 2 and 3 (Figure 3B). There were no graft failures or recipient deaths. To characterize the unique features of rejection compared with nonrejection time points in limb transplantation, we characterized circulating T cells during those time points. For the rejection events, we selected blood samples corresponding to a respective Banff grading of 2, 2/3, or 3 skin biopsies from all three individuals. For nonrejection time points, the samples revealed grade 0 or 1 (grade 1 biopsy findings are regarded as non-specific for rejection and are not treated at our institution but monitored closely over time). Compared with nonrejection time points, rejection episodes were characterized by an increase in circulating total CD8+ cells (Figures 3C and 3D), CD8+ TEMRA (Figures 3E and 3F) and CD4+ TEMRA cells (Figures 3G and 3H). Percentages of circulating Treg cells did not change during rejection (Figure 3I), whereas absolute numbers tended to be decreased at rejection time points compared with nonrejection (Figure 3J). In the peripheral blood, we observed an increase in interferon (IFN)-γ-producing CD4+ T cells (Figure 3K) and a decrease in interleukin-4 (IL-4) production by CD4+ T cells during rejection (Figure 3L). No changes were observed in production of IL-17 by circulating CD4+ T cells (Figure 3M) or IFN-γ production by CD8+ T cells (Figure 3N) during rejection events compared with nonrejection.

Molecular characterization of the graft microenvironment during rejection

To determine the molecular characteristics of the skin tissue associated with rejection, we compared the gene expression profiles of rejection (grades 2–3) and nonrejection (grade 0) events using NanoString technology. Among 770 genes analyzed, 57 genes were differentially expressed during rejection (log2 fold
change > 2; unadjusted p < 0.01). The differentially expressed genes (DEGs) are displayed in Figure 4A. A principal-component analysis (PCA) was performed for the top 57 DEGs and demonstrated separate clustering of samples with rejection compared with nonrejection events, except for one resolving rejection that clustered with the nonrejection samples (Figure 4B). We used Gene Ontology analysis (GO) to better assess the biological processes that occurred during rejection in the allograft microenvironment. Immune, inflammatory, and chemokine-related responses were strongly associated with the DEGs during the rejection events (Figure 4C). Skin biopsies with rejection had a distinct gene signature compared with nonrejection biopsies.

Rejection is characterized by expression of T cell-recruiting chemokines

From the distinctive gene signature during rejection (Figure 5A), the single most upregulated gene was CXCL13 (Figure 5B; log2 fold change = 6.1 compared with nonrejection). Following this, many of the top upregulated genes encoded for proteins associated with chemokines and chemokine-mediated signaling (CCL18, CCL17, CXCL9, and CCL5; Figure 5B), with CCL18 having a 2-fold increase during rejection. We also found increased gene expression in association with T cell co-stimulation (TNFRSF4 and CD28; Figure 5C) and effector immune molecules (GZMB, GZMA, KLRK1, and GNL5; Figure 5E). On the other hand, rejection biopsies also showed increased expression of inhibitory immune checkpoints (LAG3, CTLA4, and CD274; Figure 5D), suggesting that regulatory pathways may be triggered during rejection to counterbalance inflammatory responses.

CCL18 enhances recruitment of allogeneic T cells to human skin xenografts

Among the chemokines upregulated during rejection, CCL18 was particularly interesting, based on its primary expression in skin dendritic cells, Langerhans cells, and macrophages.12–14 CCL18 enhances recruitment of allogeneic T cells to human skin xenografts.12 Among the NSG recipients that had received PBMCs, subcutaneous injections of CCL18 into human skin xenografts led to significant macroscopic changes, including tissue shrinkage, discoloration, and dry appearance (Figure 6B). Anti-CCR8 treatment reduced the macroscopic signs of graft rejection induced by CCL18 (Figure 6B). To evaluate whether CCL18 deleterious effects on skin xenografts were dependent on the recipient’s immune system, a subgroup of animals was treated with subcutaneous injections of CCL18 or PBS 1× in the absence of PBMCs (Figure 6A). Interestingly, CCL18 treatment did not induce macroscopic changes in skin xenografts of animals that did not receive PBMCs, suggesting that CCL18 effects are dependent on the recipient’s immune system (Figure 6C). Histologically, xenografts treated with CCL18 demonstrated reduced CD31+ (vascular endothelium) staining (Figures 6B and 6D) and increased presence of CD3+ cells (Figures 6B and 6E) compared with PBS-injected animals. The anti-CCR8 treatment restored the presence of CD31+ structures (Figures 6B and 6D) and decreased CD3+ cells (Figures 6B and 6E). NSG mice that did not receive PBMCs presented the highest levels of CD31+ vessels that were not affected by CCL18 treatment (Figures 6C and 6D). Xenograft-resident human CD45+ cells were unchanged in CCL18-treated animals that did not receive PBMCs (Figures 6C and 6F). Thus, our data suggest that CCL18 can have major deleterious effects on skin xenografts, which is dependent on the presence of human immune cells.

We next assessed and quantified recruitment of T cells to the skin using T cell extraction protocols and flow cytometry. CCL18 significantly increased the numbers of CD4+ and CD8+ T cells per skin area (Figure 6G and S4). The anti-CCR8 treatment markedly decreased CCL18-induced recruitment of CD4+ and CD8+ T cells (Figure 6G). CCL18 has been shown to recruit cutaneous lymphocyte-associated antigen (CLA)+ T cells to the skin microenvironment.5 We observed enhanced numbers of CLA+CD4+ and CLA+CD8+ T cells (Figure 6H) as well as CCR8+CD4+ and CCR8+CD8+ T cells (Figure 6I) in skin allografts treated with CCL18. In contrast, CCL18-induced recruitment of CLA+ and CCR8+ T cells was reduced markedly in anti-CCR8-treated animals (Figures 6H and 6I). Last, we found that CCL18-treated skin was associated with an increased number of Th1, Th2, and Th17 CD4+ T cells compared with vehicle-treated skin, whereas anti-CCR8 treatment inhibited this recruitment (Figure 6J). Our data suggest that CCL18 is an important local chemokine that can increase recruitment of CLA+ and CCR8+ allo-T cells to human skin xenografts and accelerate graft rejection. CCR8 blockade can substantially abrogate CCL18-induced T cell recruitment and pathogenic effects in skin xenografts.
DISCUSSION

This study demonstrates that circulating immune cells from limb transplant recipients with no significant graft rejection have predominant Th2 and Treg cell phenotypes that are shifted to Th1 and CD8 responses during rejection. A similar peripheral immune profile was observed in face transplant recipients, as described previously by our group. However, despite the increased circulating Th17 cells over time, we did not observe an increase in Th17 cells infiltrating the allograft during rejection, as demonstrated in face transplant recipients. On one hand, these data suggest that, although skin is the main organ targeted by the immune system in both cohorts of individuals, the dominant effector response appears to differ. On the other hand, chronic rejection has been reported for VCA patients, and this increase in circulating Th17 cells has been associated with chronic graft injury in kidney transplant recipients, whereas reduced Th17 cells have been linked to allograft tolerance.

In our previous work, focused on face transplant rejection, we found that effector cells represented contributions from...
recipient and donor immune pools. Moreover, donor T cells in rejecting grafts exhibited resident memory phenotypes, implicating local expansion in the transplanted tissue. Targeting events appeared to involve primarily cutaneous venules as well as epithelial domains in the epidermis and hair follicles, where keratinocyte stem cells normally reside. Whether key differences will also emerge in the evolutionary immunopathology between limb and face transplant rejection is a topic in need of further study. However, it is intriguing that Th17 cell pathways are implicated in limb transplantation in the context of data showing that IL-17 can target and activate skin epithelial stem cells through the TRAF4-ERK5 axis. 

The skin is an immunologically rich tissue with more than \( \frac{10^6}{cm^2} \) resident T cells/cm² and a diverse and dynamic population of APCs. Seminal studies by Murray have suggested that the skin is the most immunogenic organ. Different skin locations are exposed to diverse external physical and chemical insults that may affect the skin microbiome and the local immune response. This exposure could cause local non-specific inflammation and mimic alloimmune injury. Through debulking surgeries, we were able to evaluate the local immune profile in the allograft microenvironment and compared it with the adjacent native skin, demonstrating higher numbers of activated T cells in non-rejecting allograft skin. The comparison with adjacent native tissue is crucial because skin from different body areas has significant variation in its immunological content. In agreement with our data, an independent study demonstrated that the cellular infiltrates in skin biopsies from hand transplant recipients were predominantly composed of T cells. Because allograft and adjacent native skin tissues were exposed to the same external factors, our data suggest a more immunologically active environment in the allograft, likely related to continuous local shedding of alloantigens that primarily trigger the adaptive immunity and potentially the innate memory alloresponse. Alternatively, it is possible that leukocyte-endothelium interactions responsible for T cell trafficking and accumulation are altered even in a homograft setting, and this important control situation requires further scrutiny to define this issue.

CCL18 is a chemokine produced by APCs from the dermis and epidermis as well as by keratinocytes, and skin-homing human T cells express CCR8. Increased levels of CCL18 have been linked to atopic dermatitis, psoriasis, allergic contact hypersensitivity, and other human chronic inflammatory diseases. In this report, we found an increase in CCL18 in skin tissue during rejection.
rejection of VCA. In a humanized skin transplantation model, we demonstrated that local CCL18 injection led to a higher infiltration of T cells in the xenografted human skin. Our results suggest that CCL18 may contribute to VCA rejection by promoting binding of CLA+ T cells and increasing homing of human memory T cells to the skin. Supporting this possibility, we also observed an increase in skin-infiltrating CLA+ T cells following CCL18 treatment. These cells have been described to mainly have a memory Th1 cell phenotype and collaborate in immune surveillance of healthy skin. When activated T cells infiltrate the skin
allograft, the local inflammatory response may potentiate their effect and further drive allograft rejection. Among other chemokines present during rejection of extremity transplants, CCL18 may have a unique role in T cell recruitment to the skin in comparison with other organ transplants and thus is a potentially promising selective target for down modulation of the alloimmune response in VCA transplantation.

Identification of potential biomarkers of rejection is of paramount importance to provide additional tools to diagnose rejection, help physicians in their decision-making about treatment, and develop new therapies. Our group has previously identified serum MMP3 protein as a potential biomarker to stratify VCA recipients according to the severity of rejection. Here, besides the increase in CCL18, we also observed an increase in different chemokines, including CXCL9 and CCL5. Along the same lines as our findings, Hautz et al. have demonstrated that markers related to lymphocyte trafficking correlated with the severity of skin rejection in a cohort of five limb transplant recipients. Thus, limb transplant rejection is characterized by upregulation of lymphocyte-attracting chemokines and trafficking markers. These markers comprise potential targets for immunosuppressive drugs. Different chemokines have been reported to be upregulated and have a role during skin allograft rejection responses. CXCL9 and CCL5 have been demonstrated to be upregulated in skin allografts, but not isografts, a few days before rejection. CXCL9, CXCL10, and CCL5 are associated with kidney rejection, and their presence in the urine of transplant recipients is being explored as a rejection biomarker. This highlights the potential of chemokines to be used as biomarkers.

Our small number of individuals reflects, in part, the novelty and challenges of extremity transplantation in humans. Despite this, our study employed prospective blood and skin graft collection in combination with high-throughput technologies like NanoString to uncover unique aspects of the rejection process in extremity transplant recipients that may account for its relatively high rejection rate. This comprehensive report of limb transplant recipients is a result of the assembly and curation of a unique biobank with more than 45 time points involving surveillance and rejection episodes. It is now important to validate the pathogenic role of CCL18 and other potential T cell-attracting chemokines in other limb and VCA transplantation cohorts.

Limb transplantation is a clinically feasible procedure for amputees, and use of a solid organ transplantation-based immunosuppressive regimen has yielded good short/medium-term graft outcomes. Nonetheless, the high frequency of cellular rejection is a concerning long-term barrier. Development of novel biomarkers in larger cohorts and less toxic, narrowly targeted skin-specific immunosuppression strategies are critical to advance the field.

Limitations of the study

Limitations of our study include its single-center nature and the small number of individuals evaluated, limiting major extrapolations. Our findings need to be validated in other limb and VCA cohorts from different centers. We also acknowledged that, although NanoString is a useful tool, its detection capacity is limited to fewer than 1,000 transcripts. More comprehensive analyses, like single-cell RNA sequencing, could have identified other transcriptional pathways unique to the rejection process in limb transplant recipients. Finally, the results from the humanized skin transplantation are limited by the variability found in this model and the inability to fully recapitulate the complexity of the human immune system.

STAR+METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100559.

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AUTHOR CONTRIBUTIONS

T.J.B. and L.V.R. conceived the study and wrote the manuscript. T.J.B. performed flow cytometry, NanoString, animal experiments, and analyses. P.A., G.F.M., and G.C.L. performed the patient histology experiments and analyses. B.K. collected the clinical data. R.B.G., M.L.-F., and B.T.A. assisted T.J.B. with the animal transplants. D.G. performed the xenograft histology. B.P., G.F.M., R.A.C., S.A.I., G.C.L., and S.G.T. helped interpret the results and edited the manuscript. All authors reviewed the manuscript critically for important intellectual content and gave final approval of the version to be submitted.
DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community.

REFERENCES

1. Murray, J.E. (1971). Organ transplantation (skin, kidney, heart) and the plastic surgeon. Plast. Reconstr. Surg. 47, 425–431.
2. Kollar, B., Pomahac, B., and Riella, L.V. (2019). Novel immunological and clinical insights in vascularized composite allograft transplantation. Curr. Opin. Organ Transpl. 24, 42–48.
3. Hein, R.E., Ruch, D.S., Klifto, C.S., Leversedge, F.J., Mithani, S.K., Pidgornyi, T.S., Richard, M.J., and Cendales, L.C. (2019). Hand transplantation in the United States: a review of the organ procurement and transplantation network/united network for organ sharing database. Am. J. Transpl. 20, 1417–1423.
4. Islam, S.A., Ling, M.F., Leung, J., S preffer, W.G., and Luster, A.D. (2013). Identification of human CCR8 as a CCL18 receptor. J. Exp. Med. 210, 1889–1898.
5. Gunther, C., Bello-Fernandez, C., Kopp, T., Kund, J., Carballido-Perrig, N., Hinteregger, S., Fassl, S., Schwarzzer, C., Lametschwandtner, G., Stingl, G., et al. (2005). CCL18 is expressed in atopic dermatitis and mediates skin homing of human memory T cells. J. Immunol. 174, 1723–1728.
6. Park, C.O., Lee, H.J., Lee, J.H., Wu, W.H., Chang, N.S., Hua, L., Lee, M.G., and Lee, K.H. (2008). Increased expression of CC chemokine ligand 18 in extrinsic atopic dermatitis patients. Exp. Dermatol. 17, 24–29.
7. Vieyra-Garcia, P., Crouch, J.D., O’Malley, J.T., Seger, E.W., Yang, C.H., Landin, L., Remmelink, M., Hewitt, C.W., Landgren, T., Lyons, B., et al. (2019). Benign T cell drive clinical skin inflammation in cutaneous T cell lymphoma. JCI Insight 4, e124233.
8. Weaver, C.T., Elson, C.O., Fouser, L.A., and Kolls, J.K. (2013). The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. Annu. Rev. Pathol. Mech. Dis. 8, 477–512.
9. Liu, T., Li, S., Ying, S., Tang, S., Ding, Y., Li, Y., Qiao, J., and Fang, H. (2020). The IL-23/IL-17 pathway in inflammatory skin diseases of the intestines, lungs, and skin. Annu. Rev. Pathol. Mech. Dis. 11, 2971.
10. Cendales, L.C., Konatik, J., Schneeberger, S., Burns, C., Ruiz, P., Landin, L., Remmelink, M., Hewitt, C.W., Langdron, T., Lyons, B., et al. (2008). The Banff 2007 working classification of skin-containing composite tissue allotransplantation. Am. J. Transpl. 8, 1396–1400.
11. Tasigjorgos, S., Kollar, B., Turk, M., Perry, B., Alhezti, M., Kiwanuka, H., Nizzi, M.-C., Marty, F.M., Chandraker, A., Tullius, S.G., et al. (2019). Five-Year follow-up after face transplantation. N. Engl. J. Med. 380, 2579–2581.
12. Gunther, C., Zimmermann, N., Berndt, N., Grosser, M., Stein, A., Koch, A., and Meurer, M. (2011). Up-regulation of the chemokine CCL18 by macrophages is a potential immunomodulatory pathway in cutaneous T-cell lymphoma. Am. J. Pathol. 179, 1434–1442.
13. He, H., Suryawanshi, H., Morozov, P., Gay-Mimbreta, J., De Luca, E., Kim, H.J., Kameyama, N., Estrada, Y., Der, E., Krueger, J.G., et al. (2020). Single-cell transcriptome analysis of human skin identifies novel fibroblast subpopulation and enrichment of immune subsets in atopic dermatitis. J. Allergy Clin. Immunol. 145, 1615–1626.
14. Reynolds, G., Vegh, P., Fletcher, J., Poyner, E.F.M., Stephenson, E., Goh, I., Botting, R.A., Huang, N., Olabi, B., Dubois, A., et al. (2021). Developmental cell programs are co-opted in inflammatory skin disease. Science 371, eaba6500.
15. Borges, T.J., O’Malley, J.T., Wo, L., Murakami, N., Smith, B., Azzi, J., Tripathi, S., Lane, J.D., Bueno, E.M., Clark, R.A., et al. (2016). Codominant role of interferon-γ and interleukin-17-producing T cells during rejection in full facial transplant recipients. Am. J. Transpl. 16, 2158–2171.
16. Kreizdon, N., Lian, C.G., Wells, M., Wo, L., Tasigjorgos, S., Xu, S., Borges, T.J., Frierson, R.M., Stanek, E., Riella, L.V., et al. (2019). Chronic rejection of human face allografts. Am. J. Transpl. 19, 1168–1177.
17. Chung, B.H., Kim, K.W., Kim, B.-M., Doh, K.C., Cho, M.-L., and Yang, C.W. (2015). Increase of Th17 cell phenotype in kidney transplant recipients with chronic allograft dysfunction. PLoS One 10, e0145258.
18. Nova-Lamperti, E., Romano, M., Christakoudi, S., Runglall, M., McGregor, R., Mobillo, P., Kamra, Y., Tsui, T.-L., Norris, S., John, S., et al. (2018). Reduced TCR signaling contributes to impaired Th17 responses in tolerant kidney transplant recipients. Transplantation 102, e10–e17.
19. Lian, C.G., Bueno, E.M., Grant, S.R., Laga, A.C., Saavedra, A.P., Lin, W.M., Susa, J.S., Zhan, Q., Chandraker, A.K., Tullius, S.G., et al. (2014). Biomarker evaluation of face transplant rejection: association of donor T cells with target cell injury. Mod. Pathol. 27, 788–799.
20. Wu, L., Chen, X., Zhao, J., Martin, B., Zepp, J.A., Ko, J.S., Gu, C., Cai, G., Ouyang, W., Sen, G., et al. (2015). A novel IL-17 signaling pathway controlling keratinocyte proliferation and tumorigenesis via the TRAF4-ERK5 axis. J. Exp. Med. 212, 1571–1587.
21. Clark, R.A., Chong, B., Mirandachini, N., Brinster, N.K., Yamanaka, K.-I., Dowgier, R.K., and Kupper, T.S. (2006). The vast majority of CLA+ T cells are resident in normal skin. J. Immunol. 176, 4431–4439.
22. Heath, W.R., and Carbone, F.R. (2013). The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. Nat. Immunol. 14, 978–985.
23. Etra, J.W., Shores, J.T., Sander, I.B., Brandacher, G., and Lee, W.P.A. (2020). Trauma-induced rejection in vascularized composite allotransplantation. Ann. Surg. 271, e113–e114.
24. Lodrup, R.G., Turk, M., Win, T.S., Marty, F.M., Molway, D., Tullius, S.G., Pomahac, B., and Tabot, S.G. (2017). Seasonal variability precipitating hand transplant rejection? Transplantation 101, e313.
25. Tong, P.L., Roediger, B., Kolesnikoff, N., Bro, M., Tay, S.S., Jain, R., Shaw, L.E., Grimbaldston, M.A., and Weninger, W. (2013). The skin immune surveillance T Cells. J. Exp. Med. 210, 2158–2171.
26. Hautz, T., Zeiger, B., Grahammer, J., Krapf, C., Anberger, A., Brandacher, G., Landin, L., Pratschke, J., Margreiter, R., and Schneeberger, S. (2010). Molecular markers and targeted therapy of skin rejection in composite tissue allotransplantation. Am. J. Transpl. 10, 1200–1209.
27. Dai, H., Lan, P., Zhao, D., Abou-Daya, K., Liu, W., Chen, W., Friday, A.J., Williams, A.L., Sun, T., Chen, J., et al. (2020). PIRs mediate innate myeloid cell memory to nonself MHC molecules. Science 368, 1122–1127.
28. Schaefer, P., Ebert, L., Willmann, K., Blaser, A., Roos, R.S., Loetscher, P., and Moser, B. (2004). A skin-selective homing mechanism for human immune surveillance T Cells. J. Exp. Med. 199, 1265–1275.
29. Kim, H.O., Cho, S.I., Chung, B.Y., Ahn, H.K., Park, C.W., and Lee, C.H. (2012). Expression of CCL1 and CCL18 in atopic dermatitis and psoriasis. Clin. Exp. Dermatol. 37, 521–526.
30. Goebeler, M., Trautmann, A., Voss, A., Bröcker, E.B., Toksoy, A., and Gilitzer, R. (2001). Differential and sequential expression of multiple chemokines during elicitation of allergic contact hypersensitivity. Am. J. Pathol. 158, 431–440.
31. Schuyler, E. (2005). Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. J. Leukoc. Biol. 78, 14–26.
32. Colantonio, L., Iellem, A., Sinigaglia, F., and D’Ambrosio, D. (2002). Skin-homing CLA+ T cells and regulatory CD25+ T cells represent major
subsets of human peripheral blood memory T cells migrating in response to CCL1/I-309. Eur. J. Immunol. 32, 3506–3514.

33. Kollar, B., Shubin, A., Borges, T.J., Tasigiorgos, S., Win, T.S., Lian, C.G., Dillon, S.T., Gu, X., Wyrobnik, I., Murphy, G.F., et al. (2018). Increased levels of circulating MMP3 correlate with severe rejection in face transplantation. Sci. Rep. 8, 14915.

34. Kollar, B., Uffing, A., Borges, T.J., Shubin, A.V., Aoyama, B.T., Dagot, C., Haug, V., Kauke, M., Safi, A.F., Talbot, S.G., et al. (2019). MMP3 is a non-invasive biomarker of rejection in skin-bearing vascularized composite allotransplantation: a multicenter validation study. Front. Immunol. 10, 2771.

35. Watarai, Y., Koga, S., Paolone, D.R., Engeman, T.M., Tannenbaum, C., Hamilton, T.A., and Fairchild, R.L. (2000). Intraallograft chemokine RNA and protein during rejection of MHC-matched/multiple minor histocompatibility-disparate skin grafts. J. Immunol. 164, 6027–6033.

36. Hricik, D.E., Nickerson, P., Formica, R.N., Poggio, E.D., Rush, D., Newell, K.A., Goebel, J., Gibson, I.W., Fairchild, R.L., Riggs, M., et al. (2013). Multicenter validation of urinary CXCL9 as a risk-stratifying biomarker for kidney transplant injury. Am. J. Transpl. 13, 2634–2644.

37. Ho, J., Schaub, S., Wiebe, C., Gao, A., Wehmeier, C., Koller, M.T., Hirsch, H.H., Hopfer, H., Nickerson, P., and Hirte-Minkowski, P. (2018). Urinary CXCL10 chemokine is associated with alloimmune and virus compartment-specific renal allograft inflammation. Transplantation 102, 521–529.

38. Kaminski, M.M., Alcantar, M.A., Lape, I.T., Greensmith, R., Huske, A.C., Valeri, J.A., Marty, F.M., Kla¨mbt, V., Azzi, J., Akalin, E., et al. (2020). A CRISPR-based assay for the detection of opportunistic infections post-transplantation and for the monitoring of transplant rejection. Nat. Biomed. Eng. 4, 601–609.

39. Christofidou-Solomidou, M., Longley, B.J., Whitaker-Menezes, D., Albeida, S.M., and Murphy, G.F. (1997). Human skin/SCID mouse chimeras as an in vivo model for human cutaneous mast cell hyperplasia. J. Invest. Dermatol. 109, 102–107.

40. Watanabe, R., Gehad, A., Yang, C., Scott, L.L., Teague, J.E., Schlabach, C., Elco, C.P., Huang, V., Matos, T.R., Kupper, T.S., et al. (2015). Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. Sci. Transl. Med. 7, 279ra39.

41. Racki, W.J., Covassin, L., Brehm, M., Pino, S., Ignotz, R., Dunn, R., Laning, J., Graves, S.K., Rossini, A.A., Shultz, L.D., et al. (2010). NOD-⁄cid IL2r-gamma(null) mouse model of human skin transplantation and allograft rejection. Transplantation 89, 527–536.

42. Borges, T.J., Murakami, N., Machado, F.D., Murshid, A., Lang, B.J., Lopes, R.L., Bellan, L.M., Uehara, M., Antunes, K.H., Pérez-Saáez, M.J., et al. (2018). March1-dependent modulation of donor MHC II on CD103+ dendritic cells mitigates alloimmunity. Nat. Commun. 9, 3482.

43. Metsalu, T., and Vilo, J. (2015). ClustVis: a web tool for visualizing clustering of multivariate data using principal component analysis and heatmap. Nucleic Acids Res. 43, W566–W570.

44. Mi, H., Muruganujan, A., Ebert, D., Huang, X., and Thomas, P.D. (2019). PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Res. 47, D419–D426.
### Key Resources Table

| REAGENT                              | SOURCE                          | IDENTIFIER                                      |
|--------------------------------------|---------------------------------|-------------------------------------------------|
| **Biological Samples**               |                                 |                                                 |
| PBMCs from limb transplant patients  | This study (BWH)                | N/A                                             |
| Punch skin biopsies from limb transplant patients | This study (BWH) | N/A                                             |
| Human discarded foreskin specimens  | This study (BWH)                | N/A                                             |
| PBMCs from healthy volunteers        | This study (BWH and MGH)        | N/A                                             |
| **Experimental models**              |                                 |                                                 |
| NSG (M. musculus)                    | Jackson Lab                     | NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ |
| **Antibodies**                       |                                 |                                                 |
| APC/Cyanine7 anti-human CD4          | Biolegend                       | Clone OKT4; RRID: AB_2687202                     |
| PerCP/Cyanine5.5 anti-human CD4      | Biolegend                       | Clone OKT4; RRID: AB_1186122                     |
| PerCP/Cy5.5 anti-human CD127         | Biolegend                       | Clone A019D5; RRID: AB_10900253                  |
| FITC anti-human CD185 (CXCR5)        | Biolegend                       | Clone J252D4; RRID: AB_2561896                   |
| PE/Cyanine7 anti-human CD279 (PD-1) | Biolegend                       | Clone EH12.2H7; RRID: AB_2159325                 |
| APC anti-human FOXP3                 | ThermoFisher                    | Clone 236A/E7; RRID: AB_10804651                 |
| Brilliant Violet 510<sup>TM</sup> anti-human CD8 | Biolegend | Clone SK1; RRID: AB_2564623                       |
| BUV737 anti-human CD8                | BD Biosciences                  | Clone SK1; RRID: AB_2870085                      |
| APC anti-human CD45RA                | BD Biosciences                  | Clone H100; RRID: AB_314416                      |
| APC anti-human CD45RA                | Biolegend                       | Clone H100; RRID: AB_314416                      |
| PE anti-human CD45RA                 | Biolegend                       | Clone H100; RRID: AB_314412                      |
| FITC anti-human CD183 (CXCR3)        | Biolegend                       | Clone G025H7; RRID: AB_10983066                  |
| PerCP/Cyanine5.5 anti-human CD197 (CCR7) | Biolegend                   | Clone G043H7; RRID: AB_10915275                 |
| PE/Cyanine7 anti-human CD196 (CCR6)  | Biolegend                       | Clone G034E3; RRID: AB_10916518                  |
| APC anti-human CD196 (CCR6)          | Biolegend                       | Clone G034E3; RRID: AB_10915987                  |
| PE anti-mouse/human B220             | Biolegend                       | Clone RA3-6B2; RRID: AB_312992                   |
| APC-eFluor 780 anti-human IFN-gamma  | Thermo Fisher                   | Clone 4S.B3; RRID: AB_10855011                   |
| PE anti-human IL-17A                 | Thermo Fisher                   | Clone eBio64DEC17; RRID: AB_1724136              |
| PE anti-human CD25                   | BD Biosciences                  | Clone M-A251; RRID: AB_2561860                   |
| PE/Cy7 anti-human CD45 Antibody      | BD Biosciences                  | Clone HI030; RRID: AB_314403                     |
| PerCP/Cyanine5.5 anti-human/mouse CLA| Biolegend                       | Clone HECA-452; RRID: AB_2565765                 |
| APC anti-human CD198 (CCR8)          | Biolegend                       | Clone L263G8; RRID: AB_2820018                   |
| Pacific Blue anti-human CD19         | Biolegend                       | Clone HB19; RRID: AB_2073118                     |
| Brilliant Violet 605<sup>TM</sup> anti-human CD3 | Biolegend | Clone OKT3; RRID: AB_2565824                       |
| PE anti-human FOXP3                  | Biolegend                       | Clone 206D; RRID: AB_492986                     |
| Purified anti-human CCR8             | Biolegend                       | Clone L263G8; RRID: AB_2562613                   |
| Purified mouse IgG2a, κ              | Biolegend                       | Clone MOPC-173; RRID: AB_326546                   |
| InVivoMAb anti-mouse Ly6G/Ly6C (Gr-1)| Bio X Cell                      | Clone RB6-8C5                                   |
| FcR Blocking Reagent, human          | Miltenyi                        | Cat # 130-059-901; RRID: AB_2892112              |
| Rabbit-anti-human CD4, polyclonal    | Novus Biologicals               | Cat # NBp1-19371; RRID: AB_1641682               |
| Mouse anti-human CD8 alpha           | Abcam                           | Clone C8/1448; RRID: AB_1280806                  |
| Goat anti-rabbit IgG Antibody (H+L), Biotinylated | Vector Labs | Cat # BA-1000-1.5                                |
| Horse anti-mouse IgG Antibody (H+L), Biotinylated | Vector Labs | Cat # BA-2000-1.5                                |
| Rabbit anti-CD3                      | Roche                           | Clone 2GV6                                      |
| Rabbit Polyclonal Anti-CD31          | Abcam                           | Cat # ab28364; RRID: AB_726362                   |
| Rabbit anti-human CD45               | Cell Signaling                  | Cat # 13917S; RRID: AB_2750898                   |
| Rabbit anti-mouse CD45               | Cell Signaling                  | Cat # 70257S; RRID: AB_2799780                   |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact (lriella@mgh.harvard.edu).

**Materials availability**
This study did not generate new unique reagents.

| REAGENT | SOURCE | IDENTIFIER |
|---------|--------|------------|
| **Critical commercial assays** | | |
| nCounter® PanCancer Immune Profiling Panel | NanoString | Cat # XT-CSO-HIP1-12 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Fixable Viability Dye eFluor 780 | Thermo Fisher | Cat # 65-0865-14 |
| Zombie NIR Fixable Viability Kit | Biolegend | Cat # 423106 |
| LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit | Thermo Fisher | Cat # L34961 |
| Foxp3/Transcription Factor Staining Buffer Set | ThermoFisher | Cat # 00-5523-00 |
| Phorbol 12-myristate 13-acetate (PMA) and Ionomycin | | |
| GolgiStop | BD Biosciences | Cat # 554724 |
| Recombinant human CCL18 | Peprotech | Cat # 300-34 |
| RPMI 1640 Medium with L-Glutamine | Lonza | Cat # 12-702Q |
| BenchMark Fetal Bovine Serum | GeminiBio | Cat # 100-106 |
| Penicillin-Streptomycin Solution | Corning | Cat # 30-002-CI |
| Collagenase type I | Thermo Fisher | Cat # 17100017 |
| Dnase I | Thermo Fisher | Cat # 18047019 |
| AccuCheck Counting Beads | Thermo Fisher | Cat # PCB100 |
| Target Retrieval Solution, Citrate pH 6 | Dako | Cat # S236984-2 |
| Streptavidin, Alexa Fluor 546 conjugate | Thermo Fisher | Cat # S11225 |
| Streptavidin, Alexa Fluor 647 conjugate | Thermo Fisher | Cat # S21374 |
| ProLong Gold Antifade Mountant with DAPI | Thermo Fisher | Cat # P36931 |
| RNase FFPE Kit | Qiagen | Cat # 73504 |
| nCounter Standard Master Kit | NanoString | Cat # NAA-AKIT-01 |
| **Software and algorithms** | | |
| FlowJo v 10.7.1 | FlowJo | N/A |
| Graphpad Prism v9.0 | GraphPad Software | N/A |
| nSolver Analysis Software v4.0.70 | Nanostring | N/A |
| Morpheus | https://software.broadinstitute.org/morpheus | N/A |
| ClustVis v2.0 | https://bit.cs.ut.ee/clustvis/ | Metsalau et al., 2015 |
| PANTHER v16.0 | http://pantherdb.org | Mi et al., 2019 |
| ZEN 2012 v1.1.2.0 | ZEISS | N/A |
| Illustrator v26.0.1 | Adobe | N/A |
| Photoshop v23.1.0 | Adobe | N/A |
| ImageJ v2.0.0-rc-69/1.52p | https://imagej.net | N/A |
| **Other** | | |
| Axio Imager.M2 | ZEISS | N/A |
| Fortessa X-20 | BD Biosciences | N/A |
| VENTANA BenchMark Stain System | Roche | N/A |
| nCOUNTER FLEX | NanoString | N/A |
Six-to-eight weeks-old NSG recipient mice were transplanted with a full-thickness (1 cm² section) human foreskin xenograft on dorsum using a sterile monofilament, non-absorbable suture, as previously described by a member of our group (GFM 39). Foreskins were used because they have fewer resident T cells. 40 Human skin tissues were obtained as discarded tissue from plastic surgery (MGB IRB 2016P001844 and 2019P002424). Transplanted animals were treated weekly with 100 μg of an anti-Gr1 antibody (clone RB6-8C5, Bio X Cell) to reduce local cellular infiltration, improve wound healing, and establish an intact human vasculature. 41 Four immune cells from debulking surgeries or skin xenografts were isolated, as described previously. 42 After harvesting, skin tissues were passed through 70 μm cell-strainer, washed and recovered in RPMI media (Lonza) supplemented with 20% FBS, 100 mM L-glutamine and penicillin/streptomycin at 4°C. After that, the tissues were minced into small pieces in 10% FCS-supplemented RPMI, followed by incubation in Collagenase type I (Thermo Fisher, 0.2%) and DNase I (Thermo Fisher, 30 Kunitz Units/mL) at 37°C for 2 h with shaking (350 rpm). Cells were passed through 70 μm cell-strainer, washed and recovered in RPMI media (Lonza) supplemented with 20% FBS, 100 mM L-glutamine and penicillin/streptomycin for 4h or overnight at 37°C. We quantified the total skin cell numbers using fluorescent AccuCheck Counting Beads (Invitrogen) by flow cytometry. Each skin sample had its area calculated and all data were normalized by skin area (in cm²).
samples from pre-transplant, 24 h, 1 week, 3-, 6- and 12-months post-transplant. Rejection time points included samples from 1 week to 3 years post-transplantation, and nonrejection time points from 1 month to 4 years post-transplantation. thawed PBMCs and recovered skin cells were Fc-blocked (Miltenyi) for 20 min before staining for surface markers for 30 min in FACS buffer (2% FBS in PBS 1x) on ice. Intracellular staining was performed with the Fixation/Permeabilization Kit (Thermo Fisher). For PBMC analyses over time and during rejection episodes, we used the following anti-human antibodies: anti-CD4 (1:100), anti-CD8 (1:100), anti-CD45RA (1:100), anti-CCR7 (1:20), anti-CD25 (1:66), anti-CD127 (1:50), anti-CXCR5 (1:100), anti-PD-1 (1:400), anti-CXCR3 (1:50), anti-IFN-γ (1:40), anti-IL-4 (1:40) and IL-17A (1:40). For cytokine detection, cell suspensions were incubated for 6 h with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA) plus 500 ng/mL ionomycin (Biolegend), and GolgiStop (BD Biosciences) in 10% FBS-RPMI, followed by surface staining, permeabilization, and intracellular staining. For the experiments with NSG mice, we used the following anti-human antibodies: anti-CD45 (1:100), anti-CD3 (1:100), anti-CD4 (1:100), anti-CD8 (1:50), anti-CD19 (1:200), anti-CLA (1:33), anti-CCR8 (1:33), anti-CXCR3 (1:50) and anti-CCR6 (1:40). Stained cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences) with FACSDiva software (BD Biosciences). Data were analyzed with FlowJo software (TreeStar). Viable cells were selected based on the staining with Fixable Viability Dye eFluor 780 (Thermo Fisher), Zombie NIR Fixable Viability Kit (Biolegend) or LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Thermo Fisher). Gating strategies for PBMCs analyzes were as previously described and can be found in Figures S2 and S3.

**Histology and immunofluorescence staining**

All specimens were sectored into 5 µm sections from formalin-fixed, paraffin-embedded tissue. Sections were dewaxed in xylene and rehydrated using serial ethanol baths in decreasing concentrations. Specimens were histopathologically evaluated with conventional hematoxylin and eosin (H&E) staining. Further evaluation was performed via immunofluorescence staining. Antigen retrieval was performed with citrate buffer pH 6 (Agilent Dako) in a pressure cooker (BioCare Medical) programmed at 110°C for 15 min. Sections were incubated with primary antibodies: rabbit-anti-human CD4 (Novus Biologicals) at 1:200 and mouse-anti-human CD8 (Abcam) at 1:50 overnight at 4°C. Afterward, biotinylated secondary antibodies goat-anti-rabbit (Vector) and horse-anti-mouse (Vector) were both incubated at 1:200 for 1 h at room temperature followed by Streptavidin-AF546 conjugate (Invitrogen) and Streptavidin-AF647 conjugate (Invitrogen), respectively, for 30 min at room temperature. Sections were mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen) and coverslipped. Immunofluorescence-stained sections were photographed using a fluorescence microscope (EVOS FL Auto 2, Invitrogen) and processed with ImageJ software.

**Immunohistochemistry**

All specimens were sectioned into 5 µm sections from formalin-fixed, paraffin-embedded tissue. Immunohistochemical staining was performed using an automated Ventana BenchMark Stain System (Roche). Antibodies used were ready-to-use rabbit anti-human CD3 (Roche), rabbit polyclonal anti-CD31 (Abcam) at 1:50, rabbit anti-human CD45 (Cell Signaling) at 1:500 and rabbit anti-mouse CD45 (Cell Signaling) at 1:500. Stained sections were photographed using an Axio Imager M2 microscope (Zeiss) and processed with ImageJ and Photoshop software.

**RNA extraction**

We obtained six consecutive 10 µm sections from formalin-fixed paraffin-embedded (FFPE) skin punch biopsies taken at different time points. Deparaffinization with Xylene and RNA extraction were performed in sterile 1.5 mL microcentrifuge tubes with the RNaseasy FFPE Kit (Qiagen), according to the manufacturer’s instructions. The concentration and purity of the isolated total RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher) at the Center for Advanced Molecular Diagnostics (CAMD) Research Core of the Brigham and Women’s Hospital. The absorbance ratio at 260/280 was used to determine RNA quality.

**NanoString nCounter assay for mRNA gene expression assay**

To investigate intragraft gene expression changes during rejection episodes, 18 limb allograft biopsies taken between December 2013 and November 2018 were retrieved from the pathology archive at Brigham and Women’s Hospital, Boston. We obtained a total of ten biopsies from rejection and eight from nonrejection episodes. From the 18 samples analyzed, one did not pass quality control (nonrejection) and was excluded from the analysis. For nonrejection episodes, these included five biopsies with Banff grades 0 and two samples with Banff grades 0/1. For rejection episodes, samples included two biopsies with Banff grades 2, two samples with Banff grade 2/3, and six biopsies with Banff grades 3. We then analyzed 770 genes with the NanoString nCounter PanCancer Immune Profiling Gene Expression (GX) CodeSet. Gene expression was measured on 100–200 ng of extracted RNA. Samples were processed on the NanoString nCounter Analysis System (NanoString Technologies) following the manufacturer’s instructions at the CAMD Research Core of the Brigham and Women’s Hospital. Images were processed into RCC files from two batches of two different lots of reagents.

We normalized raw gene expression counts, batch effect, background correction, data quality control and analyzed the data with the nSolver Analysis Software (Version 4.0.70). Twenty-seven reference genes (EIF2B4, PRPF38A, DXS50, MRPS5, AMMECR1L, CNOT4, CQG7, TLK2, ZNF143, DDX16, SAP130, TBP, SDHA, NOL7, ZC3H14, TMUB2, EDC, FCF1, PPIA, AGK, HDAC3, POLR2A, SF3A3, USP39, ZNF346, GUSB and MTMR14) were used for normalization. We used the quality control parameters recommended by the manufacturer.
QUANTIFICATION AND STATISTICAL ANALYSIS

For flow cytometry data, we used paired t-test for paired two group comparations. In animal experiments, we used unpaired Student’s t-test for comparison of the two independent groups. All statistical tests were two-sided with a type 1 error rate of 0.05 to determine statistical significance. Prism software was used for data analysis and drawing graphs (GraphPad Software, Inc., San-Diego, CA).

For the NanoString analyzes, differentially expressed genes (DEGs) between rejection and nonrejections samples were analyzed using the nSolver Analysis Software (Version 4.0.70). Samples were not paired in this analysis. Because of the low number of samples, a gene was considered differently expressed when the comparison between groups reached a log2 fold change > 2 and an unadjusted p-value < 0.01. The log2 fold change of genes assessed was transformed into Z-scores and a heatmap was created using Morpheus matrix visualization and analysis tool from the Broad Institute (https://software.broadinstitute.org/morpheus). Unsupervised principal component analysis of the top 57 DEGs clustering the samples with rejection compared to nonrejection events as generated using the web tool ClustVis using their default configurations. Gene Ontology terms were identified using the PANTHER tool (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org). All the 57 DEGs were used as an input with Homo sapiens as the organism and enriched for GO (biological processes) terms only. Fisher’s one-tailed test with Benjamini-Hochberg False Discovery Rate (FDR p value) for multiple testing corrections was used as statistics. A Volcano plot showing differentially expressed genes (DEGs) in rejection in relation to nonrejection was generated using nSolver Analysis Software (Version 4.0.70). Log2 fold change is represented in the X axis, and the Y axis displays –log10 of each gene’s p value.

ADDITIONAL RESOURCES

The study was registered at ClinicalTrials.gov (NCT01293214).