Published in final edited form as:

Sci Immunol. 2019 March 08; 4(33): . doi:10.1126/sciimmunol.aav5581.

Generation and persistence of human tissue-resident memory T cells in lung transplantation

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Abstract

Tissue resident memory T cells (TRM) maintain immunity in diverse sites as determined in mouse models, while their establishment and role in human tissues has been difficult to assess. Here, we investigated human lung TRM generation, maintenance and function in airway samples obtained longitudinally from HLA-disparate lung transplant recipients, where donor and recipient T cells could be localized and tracked over time. Donor T cells persist specifically in the lungs (and not blood) of transplant recipients and express high levels of TRM signature markers including CD69, CD103, and CD49a, while lung-infiltrating recipient T cells gradually acquire TRM phenotypes over months *in vivo*. Single cell transcriptome profiling of airway T cells reveals that donor T cells

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Author Contributions: M.E.S. and D.L.F. conceived and designed the study. M.O.F., E.B., and P.A.S., helped design scRNAseq experiments and performed gene library construction, read alignment, and analysis. M.E.S. and T.J.C. procured transplant samples and performed flow cytometry and imaging analysis. P.D. processed donor tissues and extraction of airway lymphocytes. T.S. and D.C. performed donor lung procurements. C.M. analyzed pathology specimens and guidance with imaging techniques. L.B., L.S., H.R., J.L.H., F.D.O., M.B., J.R.S., and S.A. assisted in participant recruitment and tissue procurement. M.S. assisted with study design. D.J.L. assisted with analytic approach and clinical interpretations.

Competing interests: The authors declare that they have no competing interests with regard to this work.

Data and materials availability: The scRNAseq data has been deposited in GEO (Accession number GSE124675).

comprise two TRM-like subsets with varying levels of expression of TRM-associated genes while recipient T cells comprised non-TRM and similar TRM-like subpopulations, suggesting de novo TRM generation. Transplant recipients exhibiting higher frequencies of persisting donor TRM experienced fewer adverse clinical events such as primary graft dysfunction and acute cellular rejection compared to recipients with low donor TRM persistence, suggesting that monitoring TRM dynamics could be clinically informative. Together, our results provide novel spatial and temporal insights into how human TRM develop, function, persist, and impact tissue integrity within the complexities of lung transplantation.

One Sentence Summary:

Human tissue resident memory T cells persist longterm in transplanted lungs and develop from infiltrating recipient T cells.

INTRODUCTION

Tissue resident memory T cells (TRM) are generated in diverse tissues following site-specific infection or antigen exposure, where they remain as non-circulating subsets with the potential to mediate rapid, *in situ* immune responses (1–6). Studies in mouse models have revealed important roles for TRM in tissue localized immunity. In mucosal and barrier sites such as the lung, skin and female reproductive tract, protective TRM can be generated following viral or bacterial infection or to locally-administered vaccines (2, 4, 7–15). Conversely, lung TRM can also be generated following inhaled allergen exposure, and mediate airway hyper-responsiveness in mouse asthma models (16, 17). These findings in mouse models indicate a key role for TRM in maintaining protection and promoting immunopathology. The generation and persistence of TRM in human tissues and their role in tissue-localized immune responses remain unclear.

In humans, subsets of memory T cells with phenotypes and transcriptional profiles homologous to mouse TRM have been identified in multiple tissues, including mucosal and barrier sites (lungs, intestines, skin), and primary and secondary lymphoid tissues (bone marrow, spleen, lymph nodes) (18–20). In healthy human lungs, TRM express markers for retention, adhesion and migration to tissues (CD69, CD103, CD49a, CXCR6), produce pro-inflammatory cytokines (IFN- γ , IL-17), and also exhibit upregulation of inhibitory molecules (PD-1, CD101) (18, 20, 21), pro-inflammatory and immunomodulatory properties. The functional role of human TRM *in vivo* has been inferred by correlative studies: the presence of TRM in tumors of the lung and breast is associated with a better prognosis (22, 23), while in skin, TRM are associated with disease pathology in psoriasis (21, 24). However, it is difficult to follow human immune responses *in situ*, particularly in internal tissues such as lung, and consequently, little is known about the generation, maintenance, and role of human TRM in health and disease.

Lung transplantation in which donor lungs are transplanted into HLA-mismatched recipients, represents a unique opportunity to study the development and maintenance of human TRM *in-vivo*. Notably, bronchoalveolar lavage (BAL) sampling of the airway (and blood) is obtained for clinical monitoring at regular intervals during the first year post-

transplant and at specific intervals thereafter, enabling prospective tracking of donor- and recipient-derived T cells as they persist or develop into TRM, respectively. It is also possible to correlate findings in patient BAL to critical clinical events such as primary graft dysfunction (PGD)(25), indicative of early lung injury that impacts graft survival, and acute cellular rejection (ACR) which can occur at all times post-transplantation(26).

Through a longitudinal analysis of blood and BAL samples from >20 lung transplant recipients, we demonstrate here that donor-derived T cells persist for more than one year post-transplantation specifically in the lung allograft as tissue resident memory T cells that are not detected in blood. In the BAL, donor lung T cells exhibit enhanced expression of multiple TRM signature markers, while recipient-derived T cells gradually express TRM markers in the months following transplantation. Single cell transcriptome profiling of BAL T cells reveals three distinct subpopulations--a mature TRM subset comprised of donor T cells, a second TRM-like subset containing donor and recipient cells, and a third non-TRM subset comprising recipient T cells-- that suggest *in situ* differentiation of TRM from tissue infiltrating T cells. Importantly, we found that long-term persistence of donor lung TRM is associated with reduced incidence of clinical events that precipitate lung injury, including PGD and ACR. Our findings demonstrate human TRM maturation and perpetuation in the lung, and suggest that TRM dynamics may be informative for monitoring clinical outcomes following transplantation.

RESULTS

Prospective analysis of T cell responses in lung transplant recipients

In this study, we investigated the dynamics of human lung TRM persistence, migration and generation in BAL and blood samples obtained longitudinally from twenty HLA-disparate lung transplant recipients (Fig. 1A, table S1). The majority of participants were male (70%), ranged in age from 27 to 73 years old (median 63) with a median lung allocation score of 49 (range; 33–91)(27); over one-half of patients (55%) underwent single lung transplantation. The most common indication for transplantation was interstitial lung disease (hypersensitivity pneumonitis (HP), sarcoidosis, idiopathic pulmonary fibrosis (IPF)), followed by cystic fibrosis and chronic obstructive pulmonary disease (COPD) (table S1). All patients received induction therapy with anti-CD25 antibody (basiliximab) and high dose steroids, and maintenance immunosuppression with tacrolimus and mycophenolate mofetil.

Donor derived T cells persist specifically within the lung allograft

We initially assessed the extent of lung allograft and peripheral blood donor T cell chimerism following transplantation in BAL and blood samples collected longitudinally from the transplant recipients. The origin of lymphocytes as donor- or recipient-derived was determined by staining for HLA discrepancies and analyzed by flow cytometry (Fig. 1B, C; fig. S1, S2). Peripheral blood macrochimerism, defined as the presence of > 4% of donor T cells (28), was observed postoperatively in 3/20 patients (15%) (Fig. 1D, left); however, the number of donor T cells in the blood based on cell counts was negligible in all transplant recipients by 2 months post-transplantation (< 0.01 donor cells/l) (Fig. 1D, right). Compared to blood, BAL samples contained high frequencies of donor-derived CD4⁺ and

CD8⁺ T cells in all recipients (20/20), with levels as high as 93% for donor CD8⁺ T cells and 81% for donor CD4⁺ T cells (Fig. 1E). Notably, of the 17 patients followed for > 1year, the majority of patients (13/17) retained significant donor T cell chimerism in the BAL at 12 months for both CD4⁺T cells (range of 0.5 – 55%) and CD8⁺T cells (0.7% - 85%) (Fig. 1E). Absolute numbers of T cells in BAL samples ranged from 688 – 34,678 cells (average 8991) due to heterogeneous volume recovery from the procedure and the fact that a portion was used for clinical monitoring. Over time post-transplantation, the CD4:CD8 subset composition was stably maintained for recipient T cells, but decreased significantly for donor T cells (Fig. 1F), suggesting biased persistence of donor CD8⁺T cells. Together, these results establish that donor T cells persist specifically in the lung tissue allograft.

T cells in healthy BAL exhibit predominant tissue resident memory phenotypes similar to lung parenchyma and airways

The biased persistence of donor T cells within the BAL suggested the presence of tissue resident memory T cells, which have been shown in mice and humans to predominate in mucosal and barrier tissue sites (1, 4, 29, 30). However, it was first important to determine the subset composition and anatomic origin of BAL T cells derived from healthy lungs, to establish a baseline control from which to interpret transplant patient BAL. We therefore characterized T cells derived in parallel from the BAL, lung parenchyma, and airways from healthy lungs (Fig. 2A) obtained through an organ donor tissue resource we have extensively characterized and validated (18, 30, 31). BAL T cells contained a CD4:CD8 content similar to that in the lung parenchyma but higher to that derived from the airways (Fig. 2B), and consisted of predominantly effector memory T cells (TEM; CCR7⁻CD45RA⁺) similar to lung airways and parenchyma (Fig. 2C). Lung-derived CD8⁺ T cells also contained a substantial number of terminal effector (TEMRA; CD45RA⁺CCR7⁻) cells (Fig. 2C). These findings establish that BAL T cells are predominantly memory-phenotype, similar to T cells in the lung parenchyma and airways.

We examined whether BAL memory T cells exhibited the canonical phenotypic TRM markers, including the activation/retention marker CD69 (for CD4⁺ and CD8⁺TRM) and the integrin CD103 (for CD8⁺TRM) (3, 18, 20). The majority (60->90%) of CD4⁺ and CD8⁺TEM cells in BAL express CD69 similar to frequencies in the lung and airways (Fig. 2D) suggesting that BAL T cells are predominantly TRM. CD103 is co-expressed by CD69⁺CD8⁺T cells in BAL, airway and lung, with the highest frequency of CD103 expressing CD8⁺TRM in the airways followed by BAL and lung (Fig. 2D). CD103 was also expressed by a low frequency of CD69⁺CD4⁺TRM in the airways, and at lower frequencies in the lung and BAL (Fig. 2D). Together, these finding indicate that the overwhelming majority of both CD4⁺ and CD8⁺ T cells obtained from human BAL are TRM.

Donor and recipient BAL T cells exhibit features of resting memory T cells with effector potential

Examination of the subset composition of donor and recipient BAL T cells over time post-transplantation revealed that similar to control BAL, the TEM subset predominated for donor and recipient populations, with some variations over time post-transplantation particularly among recipient CD8⁺T cells (Fig. 3A,B). Donor CD4⁺ and CD8⁺T cells were

predominantly TEM at all times post-transplantation with significant frequencies (10–30%) of TEMRA cells present among donor CD8⁺T cells (Fig. 3B, left). Recipient T cells similarly contained majority populations of TEM cells; however, CD8⁺TEMRA cells were present in substantial frequencies (20–50%) in the first 6 months post-transplantation and this frequency was reduced to the level found in donor T cells at later times (>6 months) post-transplantation (Fig. 3B, right). Given these dynamic changes in the recipient T cell subset composition, we investigated whether donor and/or recipient T cells exhibited markers of activation *in vivo*, such as HLA-DR which is upregulated on human T cells following TCR/CD3 stimulation. At early times (<1 month) post-transplantation, HLA-DR was expressed by both donor and recipient T cells; however, after one month *in vivo*, T cells of both donor and host origin were uniformly HLA-DR-negative (Fig. 3C), suggesting that T cells persisting in transplanted lungs were not overtly activated.

Functionally, both donor and recipient BAL T cells exhibited rapid production of multiple cytokines following stimulation that is a hallmark of memory T cells, and similar to the functional profiles of memory T cells isolated from healthy human lungs and lymphoid tissues (32–34). High frequencies of donor and recipient CD4⁺ and CD8⁺T cells produced IFN- with higher frequencies produced by donor compared to recipient T cells in many patients (Fig. 3D). IL-17 was produced at higher frequencies by donor compared to recipient BAL CD4⁺T cells (Fig. 3E), while both donor- and recipient-derived T cells produced comparable levels of IL-2 following stimulation (Fig. 3F). Granzyme B production was higher among recipient- compared to donor-derived CD8⁺T cells (Fig. 3G). These results indicate that persisting donor- and recipient derived T cells in the lung maintain multifunctional profiles associated with mucosal memory T cells.

Tregs have been shown to play key roles in allograft acceptance in murine models (35, 36). We therefore assessed Treg frequency at early and late times post-transplantation in blood and BAL from the patients in Fig. 1 and additional patients with long-term transplants (table S2) based on gating for CD4⁺CD25⁺CD127^{lo}Foxp3⁺ T cells (37) (fig. S3). In the blood, Treg frequency in transplant recipients was lower than in control blood (fig. S3A,B), consistent with all patients having received basiliximab (anti-CD25 antibody) induction, known to cause peripheral Treg depletion(38). In the BAL, Tregs were present in much lower frequencies than found in control BAL, and largely derived from recipient T cells with negligible donor-derived Tregs, even in samples with significant T cell chimerism (fig. S3C, D). These findings establish that TEM cells are the predominant functional T cell subset in transplant patient BAL, with donor T cells being exclusively memory.

Differential expression of TRM markers by donor and recipient-derived T cells with time

We examined whether the predominant memory T cell population within patient BAL exhibited features of TRM cells. In BAL samples from transplant recipients, expression of TRM markers CD69 and CD103 differed for donor and recipient-derived T cells, and as a function of time post-transplantation as indicated in representative and compiled patient data (Fig. 4A,B). For donor T cells, the majority of CD4⁺ and CD8⁺T cells were CD69⁺, while CD103 was co-expressed by 30–40% of donor CD4⁺ T cells and >80% of donor CD8⁺T cells at >3 months post-transplantation (Fig. 4A, B). Interestingly, the frequency of CD69⁺/

CD103⁺ donor T cells was greater than or at the upper limit compared to that of control BAL T cells (Fig. 4B; compare blue line with grey shaded rectangle). These results indicate that donor T cells in patient BAL exhibit canonical features of TRM cells, consistent with their biased maintenance in the lung but not peripheral blood.

Compared to donor BAL T cells, recipient T cell populations in the BAL expressed much lower frequencies of TRM markers at early times (2–4 weeks) post-transplantation (20–40% CD69⁺CD4⁺ and CD8⁺T cells); however, by 6 months post-transplantation, >50% of recipient BAL T cells were CD69⁺, with substantial frequencies of recipient CD8⁺T cells co-expressing CD103 (Fig. 4A,B). By 3–6 months post-transplantation, recipient BAL T cells expressed CD69 and CD103 at frequencies similar to those observed in control BAL T cells (Fig. 4B). This gradual acquisition of TRM markers by recipient-derived T cells that infiltrate the lung allograft suggest *de novo* TRM generation.

In addition to CD69 and CD103, we and others have identified additional signature markers expressed by human TRM cells (18, 21) including the collagen-binding integrin CD49a and negative regulators PD-1 and CD101(39, 40). We confirmed that these three markers were expressed in high frequencies by T cells obtained from control BAL, airways and lung parenchyma (Fig. 4C-E). In the BAL of lung transplant recipients, CD49a was expressed by a significant (>50%) frequency of donor and recipient T cells in all patients, with higher frequencies expressed by donor compared to recipient T cells (Fig. 4F). Expression of PD-1 and CD101 by donor and recipient BAL T cells was more variable between donors, with 20–80% of T cells expressing these markers, and donor CD4⁺T cells expressing significantly higher levels compared to recipient-derived CD4⁺T cells (Fig. 4G,H). Together, these results show that transplant BAL T cells express additional TRM signature markers, with increased expression by donor- compared to recipient-derived T cells.

Single cell transcriptome analysis of transplant BAL T cells reveals two TRM-like subsets

To investigate the differentiation state of donor and recipient BAL T cells, we performed single cell transcriptome profiling from two lung transplant recipients (P19, P29) at >9 months post-transplantation –a timepoint in which we could dissect the heterogeneity of recipient T cells in the context of persisting donor TRM populations. We sorted single CD3⁺T cells from patient BAL into 96-well plates (Fig. 5A; fig. S4a), followed by library prep, bar coding and RNA sequencing using Plate-seq as described (41). The results obtained were referenced to individual cell-surface protein expression including donor and recipient HLA, and TRM markers CD69 and CD103 (Fig. 5B). The analysis yielded 492 unique cells from P29 and 185 unique cells from P19; each patient's transcriptome was analyzed separately to minimize batch effects (table S3).

The scRNAseq results revealed three distinct clusters of T cells from the BAL of P29 which correlated with phenotypic and cell origin differences determined by the index sorting: Cluster 1 comprised predominantly donor CD8⁺ T cells expressing CD69 and CD103 (putative TRM); cluster 2 was mainly recipient-derived T cells lacking CD69 and CD103 expression (putative circulating TEM), and cluster 3 was a mix of donor and recipient, CD4⁺ and CD8⁺ T cells, expressing CD69 +/-CD103 (TRM-like) (Fig. 5C). The clustering of

gene expression based on expression of TRM markers and donor and recipient origin suggests distinct differentiation states could be attributed to these populations.

The heat map of gene expression from individual cells in Fig. 5D shows the top differentially expressed genes in each cluster and their level of expression in individual cells from each cluster. Cluster 1 is enriched for cells expressing genes associated with T cell effector function (*GZMA*, *NKG7*, *CCL5*, *KLRD1*, *PFN1*, *CD27*, *IL32*) and TRM differentiation including TRM markers (*ITGA1*, *CXCR6*), and the transcription factors *ZNF683* (Hobit) and *RUNX3* shown to mediate CD8⁺TRM formation in mice (42, 43). Gene expression in cluster 2 differs from that of cluster 1, with greatly reduced expression of TRM and effector-associated genes, and increased expression of genes involved in regulation of transcription/translation (*RPL13*, *PABPC1*, *MLLT3*), cell cycle (*BTG1*), and cytokine signaling (*IL7R*, *JAK3*) (Fig. 5D). Cells within cluster 3 expressed TRM genes (*ITGA1*, *CXCR6*, *RUNX3*), but these were fewer in number compared to cluster 1 (Fig. 5D), and many cluster 3 cells upregulated a unique set of genes associated with cell differentiation and fate determination (*SOX11*, *CDH6*), cellular transport (*PDZD3*, *TTPAL*), cell cycle regulation (*DOCK7*) and cytokine signaling (*TNFSF13B*).

In order to determine significant differential gene expression in the clusters, we plotted the average gene expression between clusters onto a new heat map (Fig. 6A). This analysis reveals three distinct T cell subsets including two subsets with transcriptional features of TRM cells and one non-TRM subset: Cluster 1 which we designate as “mature TRM” based on the high expression of multiple TRM signature molecules and transcription factors; Cluster 3 as “TRM-like” based on differential expression of TRM-associated molecules; and cluster 2 as “TEM” based on the lack of expression of TRM signature molecules and biased recipient origin. To assess heterogeneity within each cluster, we further compared gene expression between donor- and recipient-derived T cells within each cluster (Fig. 6B). Volcano plots show a greater concordance of gene expression between donor and recipient T cells within Cluster 1 (mature TRM) with a low magnitude of differential gene expression, compared to higher magnitude of gene expression differences between donor and recipient T cells within clusters 2 and 3 (Fig. 6B). Notably, donor T cells within cluster 3 express significantly higher levels of TRM-associated genes *ITGA1*, *ZNF683* and *CXCR6* compared to recipient T cells, while recipient cells expressed higher levels of *RUNX3* and *KLRD1*. These findings suggest that cluster 2 and 3 contain cells with more heterogeneous differentiation states compared to cluster 1 (mature TRM) cells which may represent the most differentiated population.

We also obtained scRNAseq results from the BAL of P19, which contained predominantly donor-derived cells. The data resolved into two clusters by tSNE analysis (Fig. 6C) similar to the TRM-like clusters we identified for P29. Cluster 1 in P19 with high CD69/CD103 expression strongly correlated with P29 cluster 2 (Fig. 6C, D; fig. S4B). Similarly, cluster 2 for P19 correlated with cluster 3 for P29, representing a distinct TRM population (Fig. 6D). Cluster 2 from P29 representing the tissue-infiltrating TEM population was not identified in p19 (Fig. 6D), likely due to the low number of recipient T cells. Together these results confirm the presence of two TRM-like subsets in patient BAL from lung transplantation that are consistent between disparate individuals.

Persisting donor TRM localize around airways and are associated with decreased primary graft dysfunction and acute cellular rejection.

In order to gain insight into potential *in vivo* functional roles of donor TRM in the transplanted lung, we assessed their localization and association with clinical outcome. Immunofluorescence imaging of transbronchial biopsies showed large clusters of both donor and recipient derived T cells around the small airways of the allograft; described as bronchus associated lymphoid tissue (BALT) (Fig. 7). Donor and recipient-derived T cells were found to co-localize within the peribronchiolar region of the airway of these tissues (Figure 7A-I). While donor-derived CD4⁺ and CD8⁺ T cells were found exclusively within the peribronchiolar region of the airway, recipient-derived T cells were also found in the peribronchiolar, subepithelial, and intraepithelial regions. These findings indicate broader localization of recipient compared to donor T cells.

We investigated whether persistence of donor T cells in the BAL was associated with specific clinical events post-transplantation. Primary graft dysfunction (PGD), characterized by allograft opacification (on chest x-ray) within 72 hours of transplantation not secondary to infection or cardiac dysfunction, has been significantly correlated to increased chronic lung allograft dysfunction (CLAD) and reduced lung allograft survival (25, 44). Among the recipients, patients who did not experience PGD (13/20) all exhibited higher levels of donor CD4⁺ and CD8⁺ T cell chimerism in the BAL, compared to patients experiencing PGD (7/20); this difference persisted throughout the 15 month study (Fig. 8A). These results show a clear association between increased retention of donor lung TRM and reduced incidence and severity of PGD.

ACR is another major clinical complication in lung transplantation (26), diagnosed as perivascular infiltrate of T cells found on transbronchial biopsies. Analysis of ACR diagnoses at each timepoint where BAL samples were obtained from the 20 patients we followed over the 15 month study period (95 BAL samples total), showed rejection episodes in 7/20 patients with ten episodes of biopsy proven ACR, grade A1 or higher, while the remaining 13 patients did not show evidence of ACR. At all timepoints, samples from patients with ACR had significantly lower levels of donor T cell chimerism compared to levels in samples from patients without ACR which were markedly higher ($p < 0.01$, Fig. 8B). Immunofluorescence imaging from a transplant recipient with ACR showed a perivascular infiltration comprised of recipient-derived CD4⁺ and CD8⁺ T cells, and a few donor-derived HLA-A2⁺ CD4⁺ and CD8⁺ T cells sparsely distributed in the parenchymal regions (Fig. 8C-E). These results are consistent with ACR being mediated by recipient-derived T cells infiltrating the perivascular space. We also found a correlation between samples with a positive bacterial culture and low CD4⁺ T cell chimerism (Fig. 8F), but no association between BAL T cell chimerism and viral infection (fig. S5). These results show that maintenance of donor TRM is associated with improved clinical outcome, with reduce PGD and ACR, and some protective effects to infection.

DISCUSSION

The identification of TRM and their functional roles *in vivo* have been elucidated in mouse models based in part, on the ability to monitor T cell tissue-infiltration and retention *in vivo*

(19, 42, 43). Two experimental models have been used in mice to verify T cell tissue residency: parabiosis, in which primed mice are surgically conjoined to naïve mice and TRM can be identified by specific retention in the host mouse tissues, and *in-vivo* fluorescent antibody labeling to distinguish circulating T cells which become labeled from TRM that are protected from labeling (4, 29, 45, 46). The dynamics of human TRM tissue retention and development, by contrast, have been more challenging to assess.

Here, we studied human lung TRM generation, persistence and function in airway samples obtained prospectively from patients undergoing HLA-disparate lung transplantation. Using in-depth phenotypic, functional and transcriptome profiling on the single cell level, we demonstrate that donor T cells persist specifically in the lung of transplant recipients for >1 year post-transplant, express multiple TRM signature markers, and persist as two transcriptionally distinct TRM subpopulations. Recipient T cells infiltrating the lung, by contrast, are heterogeneous consisting of TRM and non-TRM populations, and exhibit increased TRM phenotypes over months *in vivo*. Notably, increased donor TRM persistence is correlated with improved clinical outcome. Together, our results provide new insights into human TRM biology in the paradigm of lung transplantation.

TRM constitute a subset of memory CD4⁺ and CD8⁺ T cells defined by their tissue retention, distinct phenotypes, transcriptional profile, and requirements for generation. Mouse studies have demonstrated a requirement for the transcription factors Hobit (encoded by the gene *ZNF683*) and Runx3 for CD8⁺TRM generation (42, 43). Human lung TRM express phenotypes and transcriptional profiles similar to mouse TRM, including upregulated expression of CD69, CD103, CD49a, and CXCR6 (18, 20, 47); expression of *ZNF683* was increased in lung CD8⁺ but not CD4⁺TRM (18). Here, we show by phenotypic, functional, and transcriptional analysis with single cell resolution that donor TRM persisting in the lung allograft express elevated levels of the major human TRM signature markers on the cell surface (CD103, CD49a, PD-1), produce IFN- γ and IL-17 when stimulated, and exhibit elevated levels of TRM-associated genes including *ZNF683* and *RUNX3*, and genes related to T cell differentiation and effector function including *NKG7*, the chemokine *CCL5*, and cytotoxic mediators *GRMA* (Granzyme A) and *PFN1* (perforin). These findings establish that persisting donor T cells are mature TRM and suggest that Hobit and Runx3 may be involved in human CD8⁺TRM generation and/or maintenance, as donor T cells were mostly CD8⁺T cells. The ability of TRM to persist at least 15 months in the donor lung and retain high functional capacity, suggests long-term tissue maintenance *in situ*. The increased expression of TRM-associated molecules by donor T cell over time, such as CD49a and CD103 which mediate interactions with collagen and epithelial cells, respectively, suggest sustained expression of these molecules are required for TRM maintenance.

Our findings from both flow cytometry and single cell RNA sequencing provide evidence that recipient T cells infiltrating the allograft from circulation developed TRM-like profiles in a stepwise fashion in the lung. Recipient TRM development occurred over months post-transplantation as assessed by a gradual increase in surface CD69 and CD103 expression reaching a steady state after 6 months. The scRNAseq results reveal two TRM populations; one with high level expression of TRM markers that was largely donor-derived and we

designate as mature TRM, and a second, donor- and recipient-derived TRM-like subset, expressing lower levels of TRM markers and higher levels of genes controlling cell fate, including the transcription factor Sox11 involved in fate determination in retinal ganglial cells (48, 49) and osteoclasts (50). A third non-TRM subset (cluster 2) comprising recipient cells may represent tissue infiltrating TEM cells not yet activated. The functional role of recipient-generated TRM as presented here is not clear; they possess the capacity for cytokine production when stimulated but do not exhibit markers of activation *in situ*, and few recipient cells are represented among mature TRM cells with high expression of effector genes.

Persisting, donor-derived, allograft T cells, often referred to as passenger lymphocytes, have been linked to clinical outcomes in liver, heart, and intestinal transplantation (28, 51, 52), although the role of circulating versus resident populations in these transplant scenarios is not clear. In heart and liver transplantation, donor T cells have been implicated in vascular pathologies and graft versus host disease, respectively (52, 53). By contrast, in intestinal transplant recipients, donor T cell chimerism in peripheral blood and intestines was linked to reduced rejection rates (28, 54). Here, we show in a cohort of 20 patients that increased persistence of donor lung TRM specifically within the allograft is associated with reduced lung injury from PGD, bacterial infections, and acute cellular rejection. Whether the donor lung TRM are acting directly to promote reduced lung injury and inflammation or serve as an indicator of a suppressed recipient immune system is not clear and requires further study in larger cohorts. Because donor lung TRM have presumably adapted to maintain protection and homeostasis in the presence of respiratory insults, they could serve similar protective functions in the transplanted lung. Localization of donor TRM near airways may facilitate their protective role to inhaled antigens, while recipient T cells disseminate throughout the lung with the potential to trigger tissue inflammation.

In summary, our results provide spatial and temporal insights into how human TRM develop, function, persist, and impact clinical outcome within the complexities of lung transplantation. Monitoring and targeting TRM persistence and localization can serve as a new strategy for promoting long-term allograft survival in the setting of lung transplantation and beyond.

MATERIAL AND METHODS

Study Design and subject recruitment:

Institutional review board (IRB) approval was obtained prior to study enrollment. Hospitalized patients who were actively listed for lung transplantation or had previously undergone lung transplantation were approached for enrollment. A total of 23 patients were recruited, of whom one died prior to transplantation, one died after transplantation but before procurement of samples and one participant had HLA similarities to their donor preventing accurate determination of cell origin. BAL and blood samples were obtained from the remaining twenty lung transplant recipients included in this study (table S1). Additional blood and BAL samples from lung transplant recipients with longer follow up time were used for assessment of Treg frequencies (table S2). All participants underwent induction immunosuppression with an IL-2 receptor antagonist (Basiliximab), high dose

glucocorticoids (methylprednisolone), mycophenolate mofetil (MMF), and tacrolimus. Maintenance immunosuppression consisted of MMF, tacrolimus, and prednisone. Seven patients experienced primary graft dysfunction (PGD), grade 1 or higher, defined as allograft opacification within 72 hours of transplantation not due to heart failure or infection. Seven patients experienced 10 discrete episodes of acute cellular rejection (ACR); the diagnosis and grading of acute cellular rejection are based on the pathologic finding of perivascular lymphocytic infiltration in transbronchial biopsy specimens.

Acquisition of control human lungs

Human lungs were obtained from brain-dead, organ donors through a collaboration with our local organ procurement organization for New York City (LiveOnNY) as previously described (31, 55). Donors ranged in age from 20 – 73 years old and the majority died of a cerebral vascular accident (table S4). BAL was obtained by placing a 25 mL pipette into the lateral basilar segment of the lower lobe of the lung, injecting 60 mL of RPMI followed by suctioning. After obtaining BAL, the same segment of lung was dissected and cells were isolated following mechanical and enzymatic digestion of the lung and airway as described (18, 31, 55, 56).

Lymphocyte isolation from BAL and peripheral blood

BAL samples were filtered through a 100 m followed by a 40 um filter, centrifuged at 1500 rpm for 5 minutes; the cell pellet was resuspended in FACS buffer for staining, or media for functional analysis and sorting. Human trypsin FcX receptor blocker (Biolegend) was then applied at room temperature for 10 minutes prior to application of antibodies. Lymphocytes were isolated from peripheral blood using lymphocyte separation media (Cellgro) and ACK lysis buffer.

Flow Cytometry Analysis

Total mononuclear cells isolated from BAL and blood were surface and intracellularly stained with fluorochrome-conjugated antibodies (table S5 for a full list of antibodies). Intracellular staining was performed after fixation and permeabilization with Invitrogen fixation/permeabilization buffer. For cytokine determination, lymphocytes were isolated from BAL and cultured for 5 hours with PMA/Ionomycin in the presence of monensin (0.4uL/200uL media, GolgiStop™ BD Biosciences), followed by treatment with fixation/permeabilization buffer and intracellular staining with anti-cytokine antibodies. Stained samples were acquired on an LSRII flow cytometer or sorted with Influx cell sorter (BD Biosciences) and data were analyzed using FCS express v6 (De Novo Software, Glendale, CA).

Immunofluorescence Imaging:

Transbronchial biopsy specimens were received from pathology in 5 m sections from paraffin. Slides were de-paraffinized using HistoClear and re-hydrated with serial dilutions of ethanol. Antigen retrieval was performed at 95 degrees for 20 minutes in the presence of DAKO pH9 Target Retrieval Solution (Agilent). Slides were pre-incubated with Blocking One buffer (Nacalai tesque, INC) followed by staining with primary antibody, washing, and

staining with secondary antibody and DAPI (table S5). Slides were imaged using an EVOS FL Auto 2 Imaging System (Thermo Fisher) and analyzed using Imaris image analysis software (Bitplane).

Single cell transcriptome profiling by RNA sequencing

Single CD3⁺T cells from patient BAL were sorted directly into wells of 96-well plates; each well contained 7.5uL lysis buffer: 0.2% Triton X-100 (Sigma), 1 U/uL SUPERaseIN (ThermoFisher), 2mM dNTPs (ThermoFisher), 2uM RT primer (Integrated DNA Technologies). Primer annealing was performed at 72°C for 3 minutes. Reverse transcription was performed by adding 7.5uL RT mix to each well (2M Betaine (Affymetrix), 2X Protoscript Buffer (New England Biolabs), 12mM MgCl₂ (ThermoFisher), 10mM DTT (ThermoFisher), 5.3U Protoscript II Reverse Transcriptase (New England Biolabs), 0.53U SUPERaseIN (ThermoFisher), 2uM Template Switching Oligo (Integrated DNA Technologies, table S6). Reverse transcription was performed at 42°C for 90 minutes, followed by 10 cycles of 50°C for 2 minutes, 42°C for 2 minutes, 70°C for 10 minutes, followed by a 4°C hold. Excess primers were removed by adding 2uL Exonuclease I (ThermoFisher) mix to each well (1.875U ExoI in water) and incubating at 37°C for 30 minutes, 85°C for 15 minutes, 75°C for 30 seconds, 4°C hold.

All wells were pooled into a single 15-ml falcon tubes and cDNA was purified and concentrated using Dynabeads[™] MyOne[™] Silane beads (ThermoFisher) according to the manufacturer's instructions. The cDNA was split into duplicate reactions containing 25uL cDNA, 25uL 2x HIFI HotStart Ready Mix (Kapa Biosystems), and 0.2M SMART PCR Primer (table S6), PCR amplified as above, and duplicate reactions were combined and purified using 0.7 volumes AMPure XP beads (Beckman Coulter). The amplified cDNA was visualized on an Agilent TapeStation and quantified using a Qubit II fluorometer (ThermoFisher).

Sequencing libraries were constructed using Nextera XT (Illumina) with modifications. A custom i5 primer was used (NexteraPCR, table S6) with 0.6ng input cDNA and 10 cycles of amplification was performed. Unique i7 indexes were used for each plate. After amplification, the library was purified with two rounds of AMPure XP beads, visualized on the TapeStation and quantified using the Qubit II fluorometer. Libraries were sequenced on an Illumina NextSeq 500 using the 75 cycle High Output kit (read lengths 26(R1) x 8(i) x 58(R2)). Custom sequencing primers were used for Read 1 (SMART_R1seq and ILMN_R1seq, see table S6). With each plate we targeted ~70M reads. Library pools were loaded at 1.8pM with 30% PhiX (Illumina).

Analysis of scRNAseq data

Read Processing: Reads were aligned to the human genome reference GRCh38 using STAR (version 2.5) (57). During alignment, reads with more than 1 mapping were recorded as unmapped using the arguments '--outFilterMultimapNmax 1' and '--outSAMunmapped Within'. Alignment counts were generated using Subread's featureCounts (version 1.6) (58) with default parameters. Reads were assigned to cell and unique molecular indicator (UMI) barcodes using UMI-tools (59).

Data preparation: Analysis of single-cell RNA-seq data was performed in R (version 3.4.4) (60). Count matrices for both patients P29 and P19 were limited to protein coding genes, based on the datasets. Cells for each patient were filtered by excluding those with extreme values of cell summary metrics: total UMI count (nUMI), number of genes detected (nGene), and proportion of expression from mitochondrial genes (PMT). This was done by modeling the distribution of each metric within each library as a normal distribution, generating probabilities for each cell using the normal distribution function, and applying cutoffs to eliminate outlier cells.

For libraries from P29, the same cutoffs were applied to each library. For nUMI, 0.1 and 0.98 were the low and high cutoffs respectively. For nGene, 0.1 and 0.98 were the low and high cutoffs respectively. For PMT, 0.94 was the high cutoff used, and there was no low cutoff applied. Each library from P19 was filtered separately due to the high degree of dissimilarity between the distributions of their cell summary metrics. The cutoffs used for each library are shown in Supplementary Table 7.

Gene Selection and Clustering

Genes used as the basis for clustering were selected in a two-step process described in detail in supplementary methods. Principal components (PCs) for clustering and visualization were selected by computationally drawing a cutoff at the inflection point in a plot of standard deviation versus PC number. The tSNE projection used in the visualizations were produced by running the t-SNE algorithm (61) as implemented in the R package tsne (62) on a Euclidean distance matrix created from a cell by cell spearman correlation matrix computed with the selected principal components.

Clustering and differential expression analysis was performed using Seurat (63). For both patients the clustering resolution used was 0.8. Differential expression analysis was performed with Wilcoxon rank-sum tests. For each cluster, differential expression analysis was performed between the cells of the cluster and all other cells. Differential expression comparisons were also made between each pair of clusters. To analyze differences across experimental factors determined during the cell sort, differential expression comparisons were made between factor values within clusters. Also, enrichment of clusters for factor values was assessed by testing the equality of proportions of a cluster for a given factor value versus all cells outside the cluster.

Comparison of clusters between patients

The clusters identified for patient 19 patient 29 were compared by assessing the similarity of the cells in each cluster from patient 19 to those in each cluster derived from patient 29. This was done by first developing a transformation matrix to score each cell's similarity to each cluster of each patient:

$$A_i = X_i^T C_i$$

Where X_i is a matrix of NEV scaled and centered across genes for patient i ; C_i is cell by cluster matrix with dummy variables for each cluster, scaled and centered column wise.

After matrices A_{19} and A_{29} were created, the columns of each were combined into one matrix, A . Then each cell's similarity to each cluster was scored as follows:

$$S = A^T X$$

Where X is a gene by cell matrix of NEV scaled and centered across rows. The matrix S is then a cluster by cell matrix containing cluster-scores. Pearson correlation coefficients were then computed between all the cells of patient 19 and all the cells of patient 29 from the values of S . The distributions of these correlation coefficients, separated by cluster, were then used to visually determine the relative similarity of clusters across patients. Heatmaps illustrating the single-cell analysis were generated in R using heatmap3 (64). Other plots were created with ggplot2 (65) and cowplot (66).

Statistical Analysis and data visualization:

All statistics were calculated using Graphpad Prism (Graphpad software, Inc., La Jolla, CA) and R (R Foundation for Statistical Computing, Vienna Austria). Statistical significance between donor and recipient cell expression of cell surface and intracellular markers was calculated using a paired t-test when occurring at a single time point. Analysis of variance (ANOVA) was used to test for statistically significant differences across T cell populations obtained from different anatomic locations among the control lung population. Unpaired t-test was used to investigate statistical significance of donor CD4⁺ and CD8⁺ T cells at 12 months between participants who experienced PGD and those who did not. The impact of PGD on T cell chimerism following transplantation for all time points was investigated using repeated measures ANOVA. Repeated measures ANOVA was used to test statistical significance between CD4⁺ and CD8⁺ T cell chimerism and the presence of ACR over time. Adobe Illustrator CC 2017 (Ventura, CA) was used to compile all graphics.

Study approval

All human studies and procedures were approved by the Columbia University Medical Center Institutional Review Board.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We would like to thank all of our study participants, the staff and transplant coordinators of LiveOnNY for their collaboration in acquisition of tissues from organ donors and the families of the organ donors for their generosity in advancing the study of human immunology.

Funding: This work was supported by NIH grants AI106697 and HL116136 awarded to D.L.F., HL114626 awarded to D.J.L., and K24 HL131937 awarded to D.J.L. M.E.S. was supported by NIH T32 HL105323, the American Society of Transplantation TIRN award and a Parker B. Francis Foundation fellowship. P.A.S. was supported by NIH R33 CA202827. P.D. was supported by a CRI-Irvington postdoctoral fellowship. Research reported in this publication was performed in the CCTI Flow Cytometry Core, supported in part by the award S10RR027050. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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Figure 1: Donor derived memory T cells persist specifically within the lung allograft.

Donor and recipient-derived T cells were evaluated in blood and BAL samples of lung transplant recipients by flow cytometry based on HLA class I disparities (see methods). (A) Schematic of experimental design to follow how donor- and recipient-derived T cells would interact in lung transplant recipients. (B) Representative flow cytometry plots of donor versus recipient CD4⁺ (left) and CD8⁺ (right) T cells derived from peripheral blood. (C) Representative flow cytometry plots show CD4⁺ (middle) and CD8⁺ (right) T cell frequency and donor/recipient origin from a representative BAL sample. (D) Left: Graphs show percent CD4⁺ (top) and CD8⁺ (bottom) T cells of donor origin (relative to total CD4⁺ or CD8⁺ T cells) in peripheral blood over time post-transplantation in individual patients (n=14 patients with > 3 samples over time). Right: Absolute cell counts of donor CD4⁺ (right top), and CD8⁺ T cells (right, bottom) in peripheral blood in the same patients, with dotted line representing average recipient T cell count over time. (E) Graphs show percent CD4⁺ (top)

and CD8⁺ (bottom) T cells of donor origin (relative to total CD4⁺ or CD8⁺T cells) in BAL samples post-transplantation, showing individual curves for each of 20 patients. Symbols for each individual patient are designated in the legend at right. (F) CD4:CD8 T cell content in the BAL of transplant recipients (total T cell content from donor and recipient) at indicated times post-transplantation. Results shown for 19 patients; significance indicated as *** (p <0.01), all other comparisons non-significant.

Figure 2: Bronchoalveolar (BAL) of human lungs samples T cells from both the lung parenchyma and airways.

(A) Schematic diagram (left) highlighting the lateral basilar segment of the lower lobe which is where the BAL was performed and where control lung and airway segments were procured, and photograph (right) of one study lung included in the analysis. (B) Representative flow cytometry plots showing CD4 and CD8 ratio across locations. Significance indicated by *** ($p = 0.0008$). (C) T cell subset composition in the BAL, airway and lung parenchyma showing effector-memory (TEM, CCR7-CD45RA⁻), terminally differentiated effector cells (TEMRA, CCR7-CD45RA⁺), central memory T cells (TCM, CCR7⁺CD45RA⁻), and naïve T cells (CCR7⁺CD45RA⁺) in representative flow cytometry plots (left) and compiled frequencies (mean±SEM) from 15 control lungs. Significance indicated by ** ($p = 0.006$). (D) Cell surface expression of tissue residency markers CD69 and CD103 by CD4⁺ and CD8⁺ T cells across locations shown as representative flow

cytometry plots (top) and compiled frequencies (bottom) from 15 donors; significance indicated by *** ($p = 0.0002$) and **** ($p < 0.0001$).

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Figure 3: Donor and recipient BAL T cells are phenotypically and functionally memory T cells. (A) Subset composition of donor and recipient CD4⁺ and CD8⁺ T cells in representative patient BAL based on CD45RA and CCR7 expression as in Fig. 2 showing frequency of TEM (red), TEMRA (blue), TCM (purple) and naïve (green). (B) Compiled data (mean $\pm$ SEM) of donor (left) and recipient (right) T cell subset composition over time post-transplantation for CD4⁺ (top) and CD8⁺ T cell (right) lineages. Results compiled from 20 patients with one sample per patient per time point. (C) HLA-DR expression by donor and recipient T cells in patient BAL at indicated times post-transplantation shown as representative flow cytometry plots (left) and compiled frequencies from 23 samples derived from 11 patients (right). For (D) through (F), T cells from patient BAL samples were stimulated with PMA/ionomycin and cytokine production determined by intracellular staining after 5 hrs. (D) IFN- γ production by donor (black) and recipient (red) CD4⁺TEM (left) and CD8⁺TEM (right) in representative flow cytometry plots and graphs showing

paired frequencies (based on percent cytokine⁺ of donor or recipient T cells) in patient BAL samples (n=8) 1–9 months post-transplantation (right). Significance indicated by * (p = 0.04) for CD4⁺ TEM and * (p = 0.01) for CD8⁺TEM. (E) IL-17 production by donor (black) and recipient (red) CD4⁺ TEM in representative flow cytometry plot (left) and graph (right) showing paired frequencies in patient BAL samples (n = 7) 1–9 months post-transplantation. Significance indicated by * (p = 0.05). (F) IL-2 production by donor (black) and recipient (red) CD4⁺ TEM in representative flow cytometry plot (left) and graph showing paired frequencies in patient BAL samples (right, n = 8); n.s., not significant. (G) Granzyme B (GzB) expression by CD4⁺ TEM (left) and CD8⁺ TEM (right) from BAL samples obtained >2 months post-transplantation in representative flow cytometry plots (left) and cumulative paired data (right) from 6 transplant recipients of donor (black) and recipient (red), significance indicated by * (p = 0.04, right).

Figure 4: Differential expression of TRM markers by donor and recipient-derived T cells with time.

(A) Expression of CD69 and CD103 by CD4⁺ (top two rows) and CD8⁺T cells (third and fourth rows) of donor or recipient origin as indicated, over time post-transplantation for one representative patient, P5. (B) Graphs show mean frequency ($\pm$ SEM) of CD69 expression (left) or CD69/CD103 co-expression (right) by CD4⁺TEM (top) or CD8⁺TEM (bottom) cells in patient BAL at indicated times post-transplantation, compiled from 20 patients. T cell origin designated as donor (blue), recipient (red) based on HLA disparities (see Figure 1); grey shaded rectangles denote one standard deviation around the average frequency of CD69⁺/CD103 expression by control BAL T cells compiled from Fig. 2D. (C) Expression of CD49a (left), PD-1 (middle) and CD101 (right) by BAL, airways and parenchyma obtained from control lungs shown as representative flow cytometry plots and mean frequencies ($\pm$ SEM) compiled from 14 lungs. Significance indicated by * ($p = 0.02$); CD49a (bottom left), significance indicated by * ($p < 0.05$) and ** ($p = 0.004$); and PD-1 (right), ns = non-significant. (D) CD49a expression by donor (black) and recipient (red) CD4⁺ TEM (top) and CD8⁺ TEM (bottom) cells in patient BAL samples shown as representative flow cytometry plots (left) and in graphs showing paired frequencies from individual patient BAL samples ($n=6$) at >1 month post-transplantation. Significance indicated as *** ($p = 0.0003$) and * ($p = 0.007$). (F) PD-1 expression by donor (black) and recipient (red) CD4⁺ TEM (top) and CD8⁺ TEM (bottom) cells in patient BAL shown as representative flow cytometry plots

(left) and graphs showing paired frequencies in patient BAL samples ($n = 14$) >6 months post-transplant. Significance indicated as *** ($p = 0.0001$); n.s., not significant. (G) CD101 expression by donor (black) and recipient (red) CD4⁺TEM (top) and CD8⁺TEM (bottom) cells in representative flow cytometry plots (left) and paired frequencies in patient BAL samples ($n=15$) at >1month post-transplantation. Significance indicated as * ($p = 0.02$); n.s., not significant.

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Figure 5: Single cell transcriptome profiling of BAL T cells reveal three distinct subsets with differential expression of TRM-associated genes.

(A) Representative flow cytometry plots from the BAL of P29 11 months post-transplantation showing gating strategy to identify cell populations sorted into 96 well plates for single cell RNA sequencing (scRNA-seq); *** indicates the population sorted: live, CD3⁺, lymphocytes, pan-HLA⁺. (B) Representative flow cytometry plot from influx sorter identifying cell surface markers indexed to individual wells. (C) Principal component clustering identifying three distinct clusters demarcated by color and t-distributed stochastic neighbor embedding (tSNE) plots visualizing cluster differentiation (see methods); indexed cell surface markers identifying cell origin (donor v recipient) and protein surface expression for CD4, CD8, CD69, and CD103 are indicated within each cluster in separate tSNE plots. (D) Heatmap illustrating single-cell analysis for top differentially expressed genes along with select genes of interest, arranged by cluster. Heatmap of z-scored expression values are

defined as $\log\left(1 + nUMI_{ij} \frac{\text{median}(nUMI_j)}{nUMI_j}\right)$ where $nUMI_{ij}$ is the number of UMI counts for gene i in cell j and $nUMI_j$ is the total number of UMI counts in cell j .

Figure 6: TRM-like subsets exhibit quantitative gene expression differences in donor and recipient T cells and are consistent between patients.

(A) Heatmap of the z-scored mean expression values (as defined in Fig. 5D) of select genes based on differential expression analysis in each cluster, with cluster 1 designated as “mature TRM”; cluster 2 designated as “TEM”, and cluster 3 designated as “TRM-like”. (B) Volcano plots showing differential gene expression in donor compared to recipient T cells in each cluster. (C) Principal component clustering from scRNA-seq analysis of CD3⁺ T cells obtained from the BAL of P19 13 months post-transplantation identifies two distinct clusters, demarcated by color; tSNE plots visualizing cluster differentiation. (D) Pearson correlation analysis comparing gene expression within clusters 0,1,2 from patient P29 to gene expression within clusters 0,1 from patient P19.

Figure 7: Donor and Recipient-derived T cells cluster near airways.

Immunofluorescence imaging of trans-bronchial biopsies (TBBx) obtained from three transplant recipients (P12, P20, P23); for P12 and P20, the recipient is HLA-A2⁺ and donor HLA-A2⁻, for P23, the recipient is HLA-A2⁻ and donor HLA-A2⁺. Short yellow arrows point to recipient derived T cells and long green arrows point to donor-derived T cells. (A,D,G) H&E stained samples showing small airway with cluster of lymphocytes. (B,E,H) Images show expression of E cadherin (purple), DAPI (blue), CD4 (green), and HLA-A2 (red); (C,F,I) Images show expression of E cadherin (purple), DAPI (blue), CD8 (green), and HLA-A2 (red).

Figure 8: Donor TRM persistence is associated with reduced clinical complications.

Patient records were examined for clinical complications including primary graft dysfunction (PGD) and episodes of acute cellular rejection (ACR) at all timepoints of BAL acquisition (see methods). (A) Graphs show percentage (mean $\pm$ SEM) of donor CD4⁺ (left) or CD8⁺ (right) T cells in the BAL over indicated times following transplantation in patients (n = 20) stratified based on those who experienced PGD (red) or did not (blue). Significance indicated by ** (left, p = 0.003) and ** (right, p = 0.002); cumulative data across all timepoints showed increased proportion of donor CD8⁺ (p = 0.008) and trends in increased donor CD4⁺ (p = 0.06) T cells in those participants without PGD (B) Donor CD4⁺ (left) and CD8⁺ (right) T cells frequencies over indicated times following transplantation in patients (n = 20 total patients; n = 7 patients with 10 discrete episodes of acute cellular rejection at any time) based on presence (filled square) or absence (empty circle) of acute cellular rejection. Significance indicated by *** (p < 0.01). (C, D, E) Serial sections of a TBBx from one

patient (P29) who experienced acute cellular rejection at one month following lung transplant (grade 1); donor is HLA-A2⁺, recipient is HLA-A2⁻. Immunofluorescence imaging stained for E cadherin (purple), CD4 (green), HLA-A2 (red), and DAPI. Yellow arrows indicate donor cells, green arrows indicate recipient cells, *identifies a blood vessel (E) H&E stain, *identifies blood vessel (*BV). (F) Frequency of donor-origin CD4 (left) and CD8 (right) T cells in the BAL stratified based on the presence or absence of a positive bacterial culture. * indicates p-value < 0.05; n.s.: not significant.

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