HHS Public Access

Author manuscript

Am J Transplant. Author manuscript; available in PMC 2017 August 01.

Published in final edited form as:

Am J Transplant. 2016 August ; 16(8): 2300–2311. doi:10.1111/ajt.13819.

Long-term persistence of donor alveolar macrophages in human lung transplant recipients that influences donor specific immune responses

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Abstract

Steady state alveolar macrophages (AM) are long-lived lung-resident macrophages with sentinel function. Evidence suggests that AM precursors originate during embryogenesis and populate lungs without replenishment by circulating leukocytes. However, their presence and persistence are unclear following human lung transplantation (LTx). Our goal was to examine donor AM longevity and evaluate whether AM of recipient origin seeds the transplanted lungs. Origin of AM was accessed using donor-recipient HLA mismatches. We demonstrate that 94–100% of AM present in bronchoalveolar lavage (BAL) were donor derived and importantly AM of recipient origin were not detected. Further, analysis of BAL cells up to 3.5 yrs post-LTx revealed that majority of AM (>87%) was donor derived. Elicitation of *de novo* donor specific antibody (DSA) is a major post-LTx complication and a risk factor for development of chronic rejection. The donor AM responded to anti-HLA framework Ab with secretion of inflammatory cytokines. Further, in an experimental murine model, we demonstrate that adoptive transfer of allogeneic AM stimulated humoral and cellular immune responses to alloantigen and lung-associated self-antigens and led to bronchiolar obstruction. Therefore, donor derived AM play an essential role in the DSA induced inflammatory cascade leading to obliterative airway disease of the transplanted lungs.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Introduction

Tissue resident macrophages (TRM) represent distinct cell populations in terms of phenotype and function (1, 2). Despite heterogeneity, TRM are adapted to their tissue of residence and participate in various tissue specific immunologic and physiologic functions (3). The requirement at lung microenvironment is unique, as during gas exchange the respiratory surface to be protected from potential airborne infectious and polluting agents. Alveolar macrophages (AM) and interstitial macrophages (IM) are two recognized lung TRM that respectively constitute 80% and 20% of lung resident macrophage pool. Further, vast majority of AM occur in the lumen of alveoli and bronchioles, and comprise up to 95% of the leukocytes present in mouse bronchoalveolar lavage (BAL) under steady state (4). Mature mouse lungs contain up to 3×10^6 AM while that of humans contain up to 6×10^9 AM (5). AM are professional phagocytes and have been implicated in homeostatic maintenance of the respiratory surface, and airway immunity and inflammation (1). Furthermore, AM are stationary cells and are found attached to the alveolar epithelium during steady state as well as inflammatory challenges (6). A selective loss of AM results in lung failure, fatal hypoxia, severe morbidity and mortality due to viral infections (7). Functional impairment of AM has been linked to pulmonary alveolar proteinosis (8). While role of AM in gaseous exchange and immune surveillance at respiratory interface has been recognized, cellular origin, persistence and/or turnover rates of AM particularly following lung transplantation (LTx) remains undefined. AM were earlier considered as a component of the lung mononuclear phagocytic system with circulating monocytes as central precursor for AM differentiation and replenishment (9, 10). A number of recent studies have examined the progenitors and phenotypes of mature TRM (reviewed in (3, 11, 12)). Opposed to the earlier held view, TRM including AM (and Kupffer cells, microglia, Langerhans cells, cardiac macrophages and osteoclasts) have prenatal origins from yolk-sac erythro-myeloid progenitors (EMP) (4, 13–17). These studies have demonstrated a negligible contribution by bone marrow (BM) derived monocytes in AM recruitment and sustenance. Further, development and differentiation of AM from EMP requires both cell-intrinsic and cell-extrinsic signals and is granulocyte macrophage colony-stimulating factor (GM-CSF) and peroxisome proliferator-activated receptor gamma dependent (2, 4, 15). AM are long-lived TRM and locally maintained independent of replenishment by BM cells (4, 18). In this background, we evaluated AM persistence following human LTx. Though, LTx remains as the viable treatment option for various end-stage lung diseases, >50% of lung allografts undergo chronic rejection in the first five years post-transplantation, clinically diagnosed as bronchiolitis obliterans syndrome (BOS). Development of immune responses to mismatched donor HLA and immune responses directed to lung-associated self-antigens are significant risk factors for development of BOS (19–22). Studies have demonstrated that depletion of donor specific antibody (DSA) as well as Abs to lung-associated self-antigens lowers the hazard ratio for the development of BOS (23–26). In view of poor long-term success following human LTx, we postulated that post-LTx persistence of donor AM can influence immunologic responses directed to donor antigens.

Materials and Methods

Collection, analysis and culture of human AM

Human LTx recipients (LTxR) that underwent bilateral LTx at Barnes Jewish Hospital/ Washington University School of Medicine were enrolled in the study. The LTxR gave informed consent and Washington University Institutional Review Board approved the studies. Recipient and donor HLA phenotypes and DSA status were obtained from histocompatibility laboratories. Cells were isolated by centrifugation of BAL fluid at 500xg for 10min at 4°C and AM were identified by flow cytometry. For AM isolation, stained live cells were sorted by a BD FACS Aria II cell sorter and cultured in RPMI 1640 supplemented with 10% FBS and 20% L-929 culture supernatant.

Functional assessment of human AM

Isolated AM were stimulated either with toll-like receptor (TLR) ligands (Human TLR 1-9 agonists, InvivoGen) or 2 phorbol 12-myristate 13-acetate (PMA) and ionomycin or mAb to framework-HLA (W6/32) or isotype control (C.1.18.4). Culture supernatants were analyzed by a 25-Plex Human Cytokine/Chemokine luminex Panel (ThermoFisher Scientific). AM endocytosis was accessed by incubation with pH sensitive pHrodoRed dextran beads and analyzed by EVOS FL Cell Imaging System (ThermoFisher Scientific).

Mice, AM reconstitution and histopathological assessment

Wild-type C57BL/6 (H-2K^b) and Balb/c (H-2K^d) mice (6–8 wks, male) were purchased from Charles River Laboratories. C57BL/6 mice that express H-2K^d transgene on macrophages (huCD68-K^d Tg C57BL/6) were described earlier (27). All animals were maintained at Washington University School of Medicine according to institutional guidelines and approved protocols. Mice were anesthetized with Ketamine/Xylazine, lungs perfused with 1×10 mL phosphate-buffered saline (PBS) via right ventricle and BAL collected via a 22G catheter installed into trachea followed by 5×1 mL washes with ice-chilled PBS supplemented with 0.5mM ethylenediamine tetraacetic acid. BAL cells were pelleted by centrifugation at 250xg for 10 min at 4°C. For reconstitution studies, AM (CD11c+ and Siglec-F+ BAL cells) were flow sorted and administered intrabronchially. Since our average yield was 6×10^5 AM (10 wk, male), we transferred 4×10^5 AM/mouse at a harvestable ratio of 60:40. Lung tissue was fixed with 10% neutral buffered formalin, paraffin embedded and sectioned for pathologic evaluations by H&E and Trichrome staining.

Detection of donor specific immune responses

Elicitation of H-2K^d specific Abs in BAL and serum following AM reconstitution were evaluated by staining mouse T cells (CD4+ T cell isolation kit, Stemcell Technologies) by BD FACSCanto II cell analyzer. Enumeration of H-2K^d and H-2K^b reactive T cells was conducted by stimulating splenocytes from recipient mice at 16 wk post AM-transfer with irradiated (30Gy) T cells from Balb/c and C57BL/6 respectively in ELISPOT assays (BD Biosciences).

Detection of lung-associated self-antigen specific immune responses

Detection of mouse *de novo* Abs to lung-associated Ags (Kα1Tubulin (Kα1T) and Collagen V (Col V)) was conducted by an ELISA assay detailed in our previous publication (28). Briefly, wells were coated with recombinant Col V or Kα1T proteins (100ng/well) and quantitation of the specific Abs in serum was done by probing with HRP conjugated goat anti-mouse Ig (Jackson Immunoresearch Laboratories). Analysis of donor specific HLA Abs, and Col V or Kα1T auto-Abs in LTx patient sera were earlier described (29). Enumeration of lung infiltrating Kα1T and Col V specific Th1 and Th17 cells was conducted by quantifying cytokine (IFN-γ and IL-17A) secreting T cells in ELISPOT assays following our optimized protocol (28).

Results

Donor and recipient HLA mismatch is associated with de novo DSA

We enrolled15 human LTxR that received first-time bilateral lung transplant where the HLA mismatches between the lung donor and LTxR encompassed at least one of the HLA-A2 or HLA-A3 allele. In this cohort, donor and recipient mismatched at >1 HLA-A locus that was correlated with development *de novo* DSA (Table 1). All of the subjects underwent an Ab directed immunosuppressive regimen following which DSA was resolved in 40% (6/15) of LTxR. Additionally, Kα1T and Col V specific Ab were detected at varying levels. Clinical history included at least one episode of acute rejection and 12 out of 15 (~70%) LTxR developed BOS.

Donor derived AM persist following human LTx

Until recently, lack of a definitive identification and characterization of AM was the major impediment to fully understand role of AM in fibrosis, immunity and inflammation of the lungs. Studies by Misharin et al. (30), and Yu et al. (31) validated by Bharat et al. (32) have utilized a set of unified surface markers to define murine and human AM respectively. Based on Bharat et al. (32), we identified human AM as larger and granular BAL cells with high auto fluorescence and being CD45+, HLA-DR+, CD15−, CD11b+, CD206+, CD169+ and CD163+ (Figure 1). This analysis eliminated lymphocytes, monocytes, neutrophils and other granulocytes that occur in clinical BAL samples. Furthermore, it allowed us to distinguish between AM from donor or recipient origin by differential HLA staining between LTxR and graft donor. Relative composition of the AM in BAL cells is presented (Table 2). Based on the above morphometric and phenotypic characteristics, AM constituted 6.87% of the total BAL cells isolated from human LTxR (Table 2) with 97% purity (Figure 1). In the LTxR cohort, donor and recipient HLA mismatches were either HLA-A2 or HLA-A3 (Table 1). We analyzed BAL samples encompassing three possible donor→recipient combinations *i.e.* A2/A3→A3/A2 (P1–P4), A2/A3→X (P5–P10) and X→A2/A3 (P11–P15) where X being a non-HLA-A2 or non-HLA-A3 allele). The analyzed BAL samples ranged between 302 to 1265 days post-LTx. Vast majority (94–100%) of identifiable AM in BAL cells from A2/A3→A3/A2 group were positive for the donor-HLA (Figure 2). Significantly, no AM carrying unique recipient HLA-A were detected. Further, in A2/A3→X group, 87–97% of the total AM population was positive for donor HLA. Likewise, we did not detect any AM in X→A2/A3 group that expressed recipient HLA. Since our probes encompassed markers for

both donor and recipient HLA in A2/A3→A3/A2, it was feasible to assess donor vs recipient contributions into the AM pool. Significantly, majority of AM in A2/A3→A3/A2 group were donor derived with no detectable contribution from the recipient. The two other groups (A2/A3→X and X→A2/A3) allowed us to estimate, respectively, the persistence of donor AM and their replacement by cells of recipient origin. Since MHC class I is ubiquitously expressed on nucleated cells and clone BB7.2 (anti-HLA-A2) and clone GAP.A3 (anti-HLA-A3) have no known cross-reactivity, we chose to focus on these two HLA-A locus mismatches. Collectively, in our experimental settings, a significant proportion (>87%) of detectable AM were donor derived indicating a long-term persistence of AM without being replaced by recipient's cells up to 3.5 years post-LTx (Figure 2).

The persistent donor derived AM respond to TLR and anti-MHC stimuli

We next determined post-LTx AM responses to various inflammatory stimuli (TLR ligands) and donor specific HLA Abs. We used highly purified (>99%) AM isolated via flow sorting to determine their responsiveness to a panel of human TLR agonists, mitogen (PMA +Ionomycin) and Abs (anti-HLA and isotype control). Cytokine production was expressed as fold increase in stimulated cells over that of unstimulated cells. Overall, AM responded to stimuli, *albeit*, differentially in range and magnitude (Figure 3). The stimuli mimicked infections by virus, gram positive and negative bacteria, and induced status of an intracellular infection. An up-regulation in inflammatory cytokines including IL-1β, IL-6, IL-12 and TNFα were prominent. Additionally, chemokines including macrophage inflammatory proteins (MIP-1α, MIP-1β), RANTES, IP-10 and MIG involved in chemotaxis of T cell and other inflammatory cells were also elicited. These cytokine/chemokine signatures are part of a broader immune response by cells including but not limited to macrophages and indicate a preservation of AM responsiveness to various infectious and inflammatory stimuli during post-LTx period. Furthermore, TLR stimuli induced IL-8 (a macrophage produced chemokine important in neutrophil recruitment and degranulation) up to 9.3 fold (Table S1). By contrast, as expected, the cytokine signature lacked any T cell specific cytokines (IL-2 and IFNγ) indicating an absence of contaminating T cells in the AM purification protocol. The most striking finding was the AM responsiveness to anti-HLA. W6/32 induced a unique signature with stimulation of IL-1β (19.47 fold), IL-6 (4.86 fold), IL-12 (8.48 fold), RANTES (4.31 fold), MIP-1α (3 fold), MIP-1β (3.3 fold), MCP-1 (3 fold), TNFα (2.75 fold) and MIG (1.88 fold) over that of isotype control (Figure 3A). Importantly, W6/32 also induced IL-8 >3 fold compared to isotype treatment (Table S1). Taken together, our data demonstrate that donor AM purified from the BAL following LTx are not only able to respond to infections but also react to MHC ligation with Abs resulting in secretion of inflammatory cytokines.

We then assessed AM endocytosis by pH sensitive dextran beads. The beads following internalization into acidic endosomal and lysosomal compartments fluoresce that allowed their detection. Following a 20-min pulsing, >95% of AM (Figure 3B) demonstrated bead uptake indicating preservation of particle endocytosis in post-LTx AM.

Adoptive transfer of AM with mismatched MHC class I induces alloimmunity in murine model

The murine AM in BAL cells were identified by being CD45+, CD11c+ and Siglec-F+ (Figure 4A). We utilized a novel huCD68-K^d Tg C57BL/6 mouse, which expresses a full length H-$2K^d$ transgene in C57BL/6 macrophages (27) to evaluate AM reconstitution. Nearly all (~98%) of AM from huCD68-Kd Tg expressed both H-$2K^d$ and H-$2K^b$ (Figure 4A). This unique expression of surface H-$2K^d$ transgene in huCD68-K^d Tg AM enabled us to examine effect of AM restricted donor specific MHC mismatches in elicitation of donor specific alloimmunity. We evaluated whether transfer of huCD68-Kd Tg (Tg) AM into C57BL/6 (B6) recipient will induce H-$2K^d$ specific immune responses. In parallel, we also carried out allogeneic transfer of B6 AM into Tg recipients and syngeneic transfer of Tg AM into Tg recipients, and transfer of B6 AM into B6 recipients. Development of H-$2K^d$ (alloantigen) reactive lung-resident T cells (Figure 4B) and alloAbs in BAL fluid and serum (Figure 4C) were analyzed. Adoptive transfer of Tg into B6 stimulated alloantigen specific T cell responses and elicited detectable H-$2K^d$ Abs both in BAL fluid as well as in serum. Since the donor AM expressed H-$2K^d$ as a transgene, it is being recognized as a foreign antigen. It is important to note that both the AM donors (huCD68-K^d Tg) and recipients (C57BL/6) had identical MHC class II (I-A^b). Therefore the donor AM was capable of priming I-A^b restricted H-$2K^d$ reactive CD4 T cells. Further, the engrafted AM expressed equivalent levels of surface MHC II present on native AM indicating an optimal antigen presentation ability (Figure 4D). Taken together, demonstration of H-$2K^d$ specific Ab and T cell responses suggest that mismatched MHC I antigen on AM is sufficient to elicit donor specific immune responses.

Allogeneic AM transfer induces small airway obstruction and fibrosis

We have earlier developed a murine obliterative airway disease model where administrations of exogenous MHC Ab into native lungs induce bronchiolitis, a clinical correlate of human BOS (33, 34). In the present study of AM transfer model, we evaluated pathologic effect of *de novo* DSA on small airway obstruction and fibrosis. Increased peribronchiolar leukocyte clusters, and greater epithelial hypertrophy and hyperplasia were evident in Tg→B6 compared to B6→Tg group (Figure 5A). Additionally, higher depositions of collagenous extracellular matrix proteins and higher occurrence of small airway obstruction in Tg→B6 mice indicated a respiratory impairment. Histopathology assessment for the AM transfer groups is presented in Figure 5B and allogeneic AM transfer (Tg→B6) that stimulated DSA resulted in a significant loss of bronchiolar architecture with concomitant increase in small airway inflammation and obstruction.

Allogeneic AM transfer induces immune responses directed to lung-associated self-antigens

In the view of Tg→B6 eliciting *de novo* DSA (Figure 4) and bronchiolar obstruction (Figure 5), we examined stimulation of lung-associated self-antigen (Kα1T and Col V) specific autoimmunity following AM transfer. Mean serum titers for anti-Kα1T and anti-Col V in Tg→B6 AM transfer mice were 105 μg/mL and 183 μg/mL respectively and were significantly higher than that in other transfer groups measured at <40 μg/mL (Figure 6A).

Furthermore, Tg→B6 stimulated significantly greater Kα1T and Col V reactive IL-17 and IFNγ secreting T cells (Figure 6B). The mean Kα1T specific IL-17 and IFNγ secreting T cells were 75 and 55 (spots/10^6 cells) respectively while those of Col V reactive T cells were 97 and 84 (spots/10^6 cells) respectively. The self-antigen reactive T cells in other AM transfer groups remained <20 spots/10^6 cells. Taken together, stimulation of T cells and development of Abs directed to lung-associated antigens indicate that MHC I mismatched AM were sufficient to induce humoral and cellular immune responses to lung-associated self-antigens.

Discussion

Despite improvements in surgical techniques and post-operative care, incidence of BOS continues to be the primary obstacle in long-term success of LTx (35). Development of *de novo* DSA to mismatched donor HLA and Ab to lung-associated self-antigens are considered major risk factors for BOS development (20, 36, 37). Role of AM in DSA induced lung inflammation and BOS is largely unknown. This is partly due to lack of a uniform experimental approach and definitive AM characterization. AM are terminally differentiated TRM and express distinctive surface markers compared to other tissue resident and circulatory macrophages (3). The recently described flow cytometric analyses (30–32) allow unambiguous identification of human and mouse AM and enable them to be distinguished from “passenger leukocytes”. It is generally believed that recipient stem cells seed the transplanted organ, and endothelial cells and macrophages repopulate the graft with potential to minimize host immune detection (39–46). However, the dichotomy of immunologic complications and poor prognosis associated with LTx intrigued us to investigate post-LTx persistence of donor AM and its contribution to chronic rejection. AM are the major lung TRM and constitute professional phagocytes at blood-air interface (47). We, for the first time, demonstrate that donor AM persist following LTx for a long period (up to 3.5 yr) and respond to HLA specific Ab resulting in secretion of pro-inflammatory cytokines. This finding has two-fold implications. First, long-term survival of donor AM (bearing donor-HLA) in transplanted lungs may serve as a repository of mismatched HLA antigens. Second, our finding demonstrating pro-inflammatory responsiveness of AM to MHC ligation indicate a putative role for AM in mediating DSA induced pathogenesis. Our findings of a long-term sustenance of AM following human LTx and our inability to detect recipient derived cellular chimerism in AM compartment is in contrast to earlier published studies (39, 40, 44, 48). The disagreement is due to differences in experimental methods and target cells studied. For example, report by Kjellström et al. (40) used DNA microsatellite described a rapid turnover of donor CD14+ BAL cells. By lineage analysis, human AM lack CD14 expression while monocytes are CD14+ (31, 49, 50). The study by Judson et al. (39) analyzed pleural cell chimerism primarily in unilateral LTx patients. Cellular composition in pleural lavage fluid includes lymphoid and myeloid cells (51) but lacks AM. Nakata et al. (44) and Kennedy et al. (48) while studying reconstitution and proliferation of BAL cells following BM transplantation (BMT) reported appearance of BM derived AM in BAL around 60 days post-BMT. These authors based their conclusions on lymphocyte-depleted monocytes and macrophages but not on purified AM following irradiation and BMT. Therefore, due to lack of a consistent analysis on AM, it is difficult to interpret these results

in recipient AM reconstitution. Our approach to identify AM is based on surface markers that enabled us to distinguish the AM from IM, monocytes, neutrophils, lymphocytes and other granulocytes (30–32). In addition, using HLA allele we are able to identify AM as either of donor or recipient origin. Until recently, AM have been consistently misidentified and misrepresented with regards to their phenotype and lineage specificity. Recent studies have concluded that yolk-sac derived fetal monocyte but not circulating hematopoietic stem cell (HSC) derived monocyte is precursor for AM development (4, 15). Genetic reconstitution and parabiosis experiments have also confirmed that circulating monocytes minimally contribute to the homeostatic AM pool (4, 15, 16, 52). Further, constitutive and inducible fate mapping of embryonic cells indicated that yolk-sac EMPs accumulated in fetal liver give rise to AM while HSC driven hematopoiesis minimally replaced the AM compartment (13). Moreover, transplantation of fetal liver cells following AM depletion by clodronate liposomes or lethal irradiation resulted in an efficient reconstitution of AM cells (53). IM, on the other hand, are blood monocyte derived and demonstrate a high turnover rate (54). The impact of an ischemia reperfusion injury (IRI) associated with LTx on AM is currently unknown. In a rat LTx model, viable AM were isolated from BAL cells following transplantation suggesting a minimal effect of IRI, if any, on AM survival (55). Though we did not specifically test the influence of IRI on AM in this study, our results suggest that human AM endured IRI stress and persisted up to 3.5 years post-LTx. While in experimental models a long-term replacement of steady state AM by HSC cells was negligible, a slow (~1yr) and partial (~40–48%) turnover of AM has been reported in BMT following myeloablative irradiation (18, 48), infection with influenza virus (56), high dose endotoxin challenge (57) and due to old age (13) that depleted the homeostatic AM pool. It remains unclear though the phenotypic and transcriptional differences between yolk-sac derived native AM and neo-differentiated AM from BM hematopoiesis. Our cohort of LTxR received no irradiation treatment and remained free from a documented viral infection during the follow-up period. So existence of a negative pressure on donor AM survival was unlikely. Further, donor AM responded to TLR agonists and MHC ligation. Responsiveness to TLR stimuli indicates AM's functional preservation as "first line of innate defense" at respiratory interface (50). Further, we demonstrate inflammatory responses by donor AM following MHC I ligation, which is in agreement with the reported dominant role for donor endothelial cells in anti-HLA induced inflammation (58). In addition, we conclusively demonstrated that the donor AM in an *ex vivo* assay stimulated inflammatory cytokines in response to anti-HLA. Extracorporeal removal of DSA has been shown to reduce inflammatory cytokines and increase anti-inflammatory cytokines (23). This has a significant clinical implication as AM may contribute to DSA induced pathogenesis in fibrotic and inflammatory lung diseases.

We investigated whether AM reconstitution is viable with adoptive transfer of allogeneic AM (with a single MHC I mismatch). Intrabronchial administration of naïve Tg AM resulted in AM persistence with a stable reconstitution rate (Figure S1). The donor AM were localized to alveoli and detectable even after four months post-transfer suggesting a viable engraftment. Since the Tg AM carried surface H-2K^{d}, an alloantigen, we evaluated whether Tg→B6 led to alloimmunity. We evaluated elicitation of local as well as systemic alloAb. Following AM transfer, Tg→B6 stimulated Ab and T cell responses to alloantigen (Figure

4B–C) and lung-associated self-antigens (Figure 6A–B) concomitant with bronchiolar obstruction and fibrosis (Figure 5A–B). AM serve as antigen presenting cells in natural infection (59) and vaccination (60, 61). Earlier studies reported persistence of donor AM and induction of alloAb (in BAL and serum) and T cells following allogeneic AM transfers (62, 63). The genetic mismatches between donor and recipient in those studies were major encompassing both MHC (I and II) and non-MHC loci. By contrast, AM transfer experiments performed in our study included one defined allelic mismatch that helped us to determine immunogenicity of MHC I mismatched AM.

A limitation to our study is narrow probe range that permitted analysis of human BAL cells only when the organ donor and recipient's genetic mismatches included at least HLA-A2 or HLA-A3. This excluded a number of potential BAL samples resulting in a small sample size. Nevertheless, our finding of donor derived AM in engrafted lungs is the first demonstration of a long-term persistence of native AM following human LTx. In this study we did not evaluate causality and/or consequence of donor AM in the development of BOS. It is possible that additional cellular and molecular processes participate in the development of DSA and bronchiolar obstruction leading to BOS. However, ability of AM to induce DSA and be stimulated by MHC Ab demonstrate an important role for AM in the DSA induced immunopathogenesis leading to chronic lung allograft rejection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from NIH (R01HL056643 and R01HL092514) and BJC foundation to TM, and a grant from NIH (R01AI102891) to MT. FZ was supported by State Scholarship Fund from China Scholarship Council (201406260136).

Abbreviations

Ab	antibody
AM	alveolar macrophages
BAL	bronchoalveolar lavage
BM	bone marrow
BMT	bone marrow transplant
BOS	bronchiolitis obliterans syndrome
Col V	Collagen V
DSA	donor specific antibody
EMP	erythro-myeloid progenitors
GM-CSF	granulocyte macrophage colony-stimulating factor

HSC	hematopoietic stem cell
IRI	ischemia reperfusion injury
IM	interstitial macrophages
Kα1T	K alpha 1 Tubulin
LTx	lung transplantation
LTxR	lung transplant recipient
PBS	phosphate-buffered saline
PMA	2 phorbol 12-myristate 13-acetate
Tg	transgenic
TLR	toll-like receptor
TRM	tissue resident macrophages

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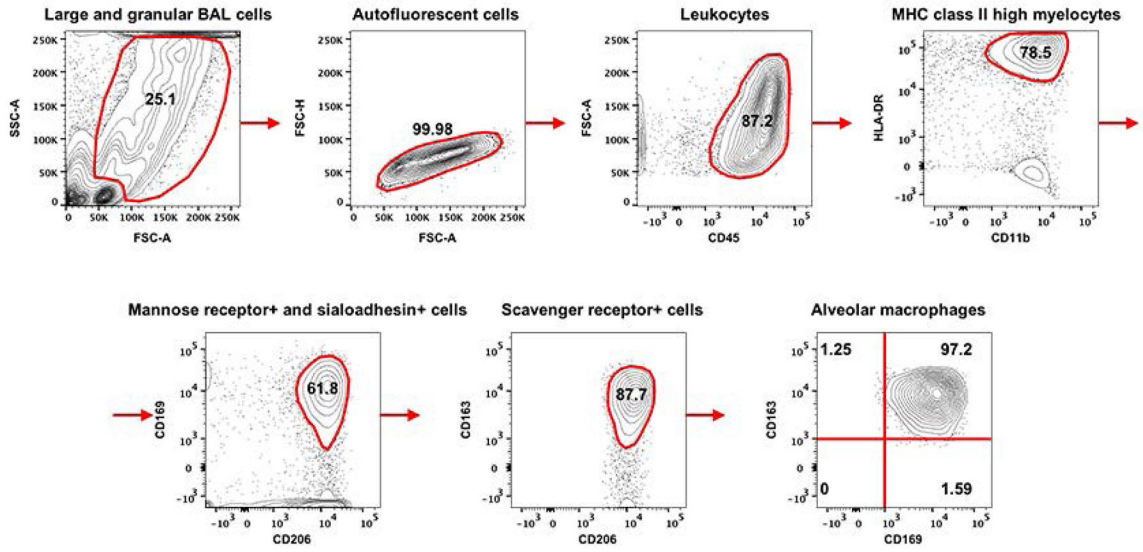

Figure 1. Detection of AM in BAL fluid from human LTxR

Strategy to identify AM by cell surface staining in a flow cytometry analysis. Freshly collected BAL cells were incubated with human BD Fc Block (BD Biosciences) and human TruStain FcX (Biolegend) to inhibit Fc specific binding followed by staining with following Abs (obtained from Biolegend and eBioscience): CD45-Alexa Fluor 488 (HI30), CD11b-PE/Cy7 (M1/70), HLA DR-APC/Cy7 (L243), CD169-APC (7-239), CD15-Brilliant Violet 510 (W6D3), CD163-Brilliant Violet 421 (GHI/61) and CD206-PE (15-2). Stained cells were fixed with neutral buffered paraformaldehyde (2%) before being analyzed by BD FACSCanto II cell analyzer (BD BioSciences) and flow data were analyzed by FlowJo 10.1 (Tree Star). Small and less-granular cells and cell clumps were excluded from analysis. Large and cells with high-granularity were selected and further enriched for high autofluorescence based of FSC and SSC criteria. The sequential flow chart of analysis is depicted. AM were defined as autofluorescent BAL cells being positive for CD45, CD11b, HLA-DR, CD169, CD206 and CD163. Frequency of cells in each gate or quadrant is indicated.

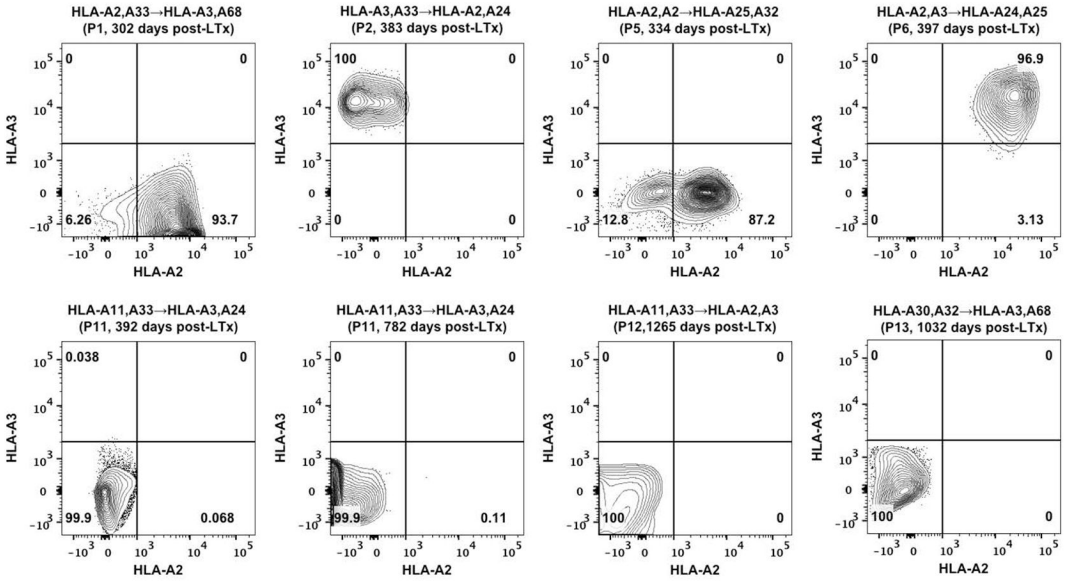

Figure 2. Persistence of donor derived AM following human LTx

Assessment of donor and recipient contribution to the post-LTx AM pool was analyzed based on donor-recipient HLA-A mismatches. AM were identified as described in Figure 1 and were further analyzed by staining with anti-HLA-A2 (BB7.2) and anti-HLA-A3 (GAP.A3) to distinguish their cellular origin. Tracking of AM in eight representative BAL specimens is presented. HLA-A mismatches (donor→recipient), patient I.D. (Table 1) and time of post-LTx BAL collection are indicated for the respective specimens. Frequency of cells in each quadrant is indicated.

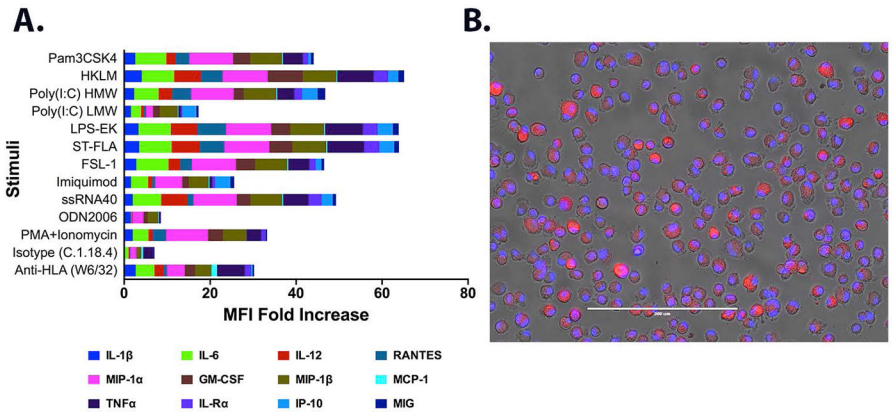

Figure 3. Preservation of AM functionality in donor derived post-LTx AM

(A) Elicitation of inflammatory cytokines and chemokines was evaluated in response to a panel of TLR agonists: TLR1/2- Pam3CysSerLys4 (Pam3CSK4); TLR2- heat-killed *Listeria monocytogenes* (HKLM); TLR3- Poly (I:C) high molecular weight 1.8–8 kb(HMW), Poly (I:C) low molecular weight 0.2–1 kb(LMW); TLR4-Lipopolysaccharide from *Escherichia coli* K12 (LPS); TLR5- *Salmonella typhimurium* Flagellin (ST-FLA); TLR6/2- N-terminal part of the 44-kDa lipoprotein LP44 of *Mycoplasma salivarium*, Pam2CGDPKHPKSF (FSL-1); TLR7- R837 (Imiquimod); TLR8-ssRNA40 and TLR9-ODN2006 type B. PMA and Inonomycin were used at 5 ng/mL and 500 ng/mL respectively and HLA specific mAb (W6/32) or isotype control (C.1.18.4) were used at 100 μg/mL to study effect of MHC I crosslinking. Flow sorted AM were seeded in a 12-well plate at 5×10^5 cells/well and release of cytokines after 24 hr stimulation at 37°C was measured in culture supernatant by a 25-plex luminex panel. The mean fluorescence intensity (MFI) of an individual analyte is expressed as fold increase in stimulated cells compared to that in un-stimulated cells. Data on 12 selected analytes from a representative AM culture are presented and computed values (pg/mL) for all (25) analytes are given in Table S1. (B) Particle endocytosis by AM was accessed by incubation with pH sensitive pHrodoRed dextran (10,000 MW) beads (50 μg/mL) for 20 min at 37°C. Cells were stained with NucBlue live ready probe to visualize nucleus and analyzed by EVOS FL Cell Imaging System. A representative merged image from bright field, and red and blue fluorescence is given. Scale bar is 200 μm.

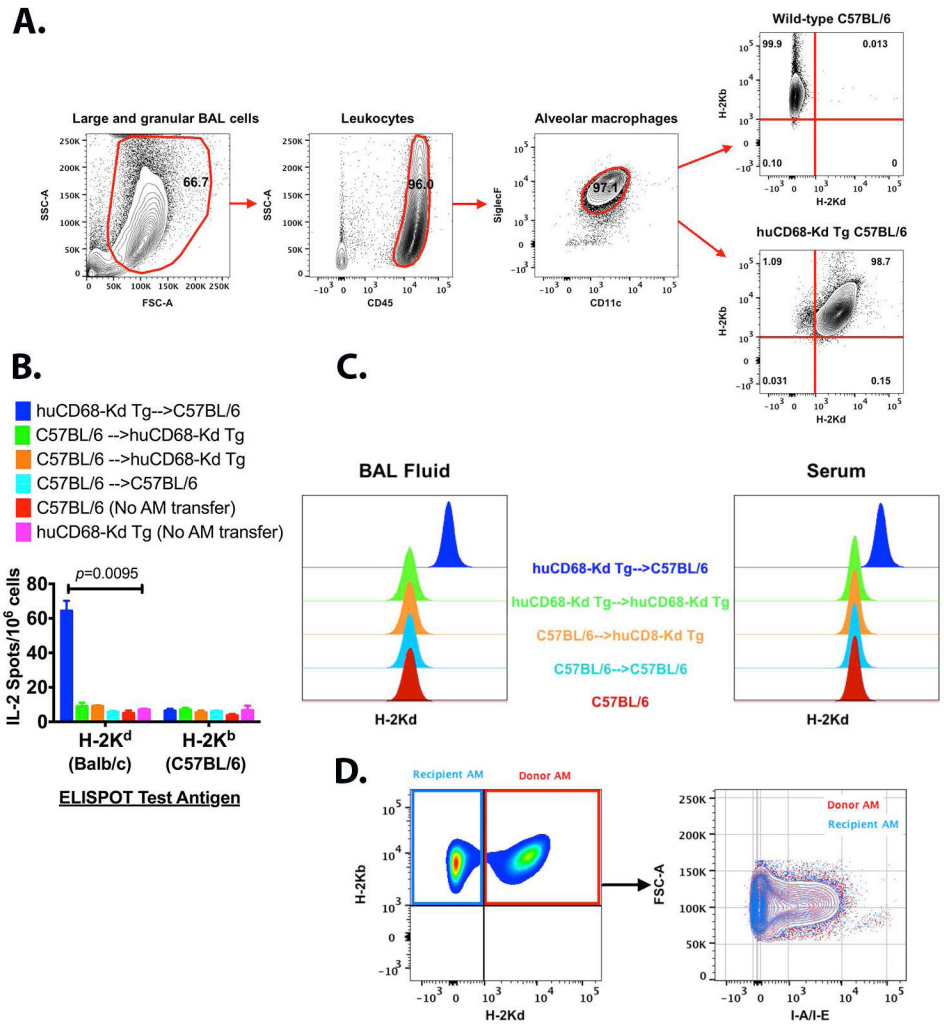

Figure 4. Induction of donor specific immunity following allogeneic AM reconstitution

(A) Gating strategy and validation of H-2K^d transgene expression on huCD68-Kd Tg C57BL/6 AM (27). The mouse BAL cells were Fc blocked (clones 2.4G2 and 93) to saturate CD16/CD32 mediated binding and were characterized by staining with CD45-PE/Cy7 (I3/2.3), CD11c-Alexa Fluor 488 (N418), Siglec-F- Brilliant violet 421 (E50-2440) and the H-2K^d transgene expression was compared by staining with H-2K^b-APC (AF6-88.5), H-2K^d-PE (SF1-1.1). Mouse AM were defined as large granular BAL cells being CD45+, CD11c+ and Siglec-F+. (B) Stimulation of allogeneic T cell response following intrabronchial AM transfer. Development of H-2K^d and H-2K^b reactive T cells was enumerated by stimulating splenocytes (1×10^6) from indicated recipient mice at 16 wk post AM-transfer with irradiated (30Gy) T cells (1×10^5) from Balb/c or C57BL/6 in a IL-2 ELISPOT assay (BD Biosciences). Five mice were used per experimental group, data represent mean±SEM and multiple t-test was applied to evaluate statistical significance (*p* value indicated). (C) Induction of H-2K^d specific Abs following AM transfer was evaluated by staining CD4+ T cells from Balb/c mice with cell free BAL fluid (neat) or serum (1:50) at 16 wk post-AM transfer or naïve mice (from panel B) followed by staining with goat anti-

mouse IgG APC. Five-six mice were used in each experiment. (D) AM reconstitution at 16 weeks post-intrabronchial transfer of huCD68-Kd Tg AM in to C57BL/6 recipients. Native AM in recipient mice were H-2K^{b+} and H-2K^{d-} while donor AM were double positive for H-2K^{b} and H-2K^{d}. Expression of MHC class II on donor and recipient AM was evaluated by staining with I-A/I-E-Brilliant Violet 421 (M5/114.15.2) and presented as overlaid image.

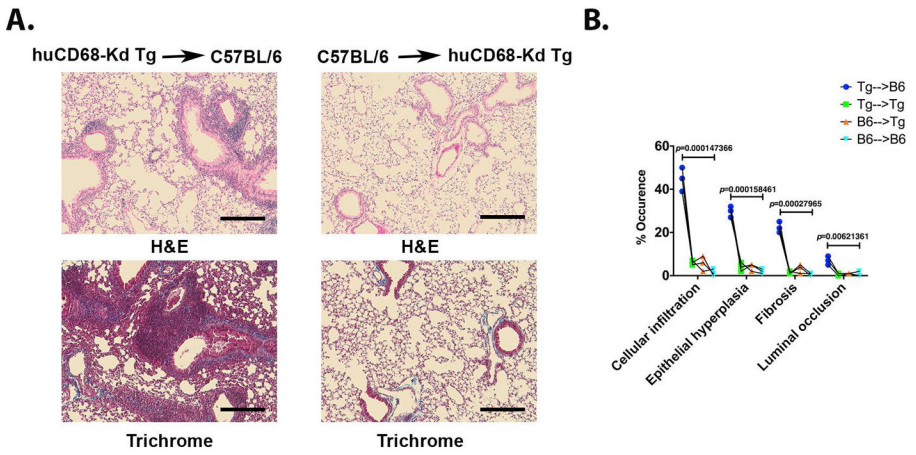

Figure 5.

Histopathologic assessment of lungs following adoptive AM transfer. (A) Paraffin embedded tissue sections were analyzed for leukocytic infiltrations (H&E) and fibrosis (Masson's Trichrome). (B) The slides were scored according to (34) and average score from five fields is presented. Three mice per group were analyzed. Multiple t-test was applied and statistical significance was determined using Holm-Sidak method (*p* value indicated). Scale bars are 40 μm.

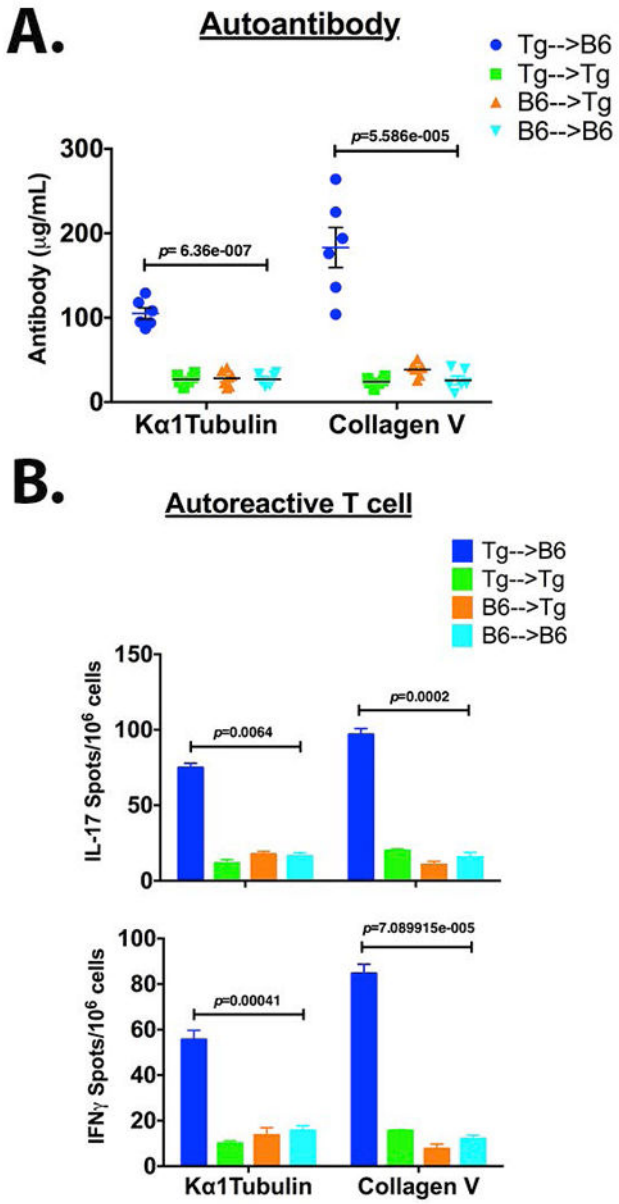

Figure 6.

Allogeneic AM reconstitution induces lung-associated antigen specific autoimmunity. (A) Elicitation of serum Ab to Kα1T and Col V at 16 wk post AM transfer was measured by ELISA (33). (B) Accumulation of Kα1T and Col V specific T cells was evaluated by IL-17A and IFNγ ELISPOT assays (BD Biosciences). Single cell suspension was prepared from lungs by digestion with Liberase TL (300μg/mL, Roche Life Science) and DNaseI (5U/mL, Sigma-Aldrich) at 37°C for 25 min. Lung leukocytes were isolated from the single cell suspension by Ficoll-Paque (Sigma-Aldrich) gradient centrifugation. One million lung leukocytes were added per well and were supplemented with 5×10^4 C57BL/6 splenocytes (irradiated at 30 Gy) as antigen presenting cells. The cells were stimulated with Col V or Kα1T protein at 1μg/mL and cytokine producing spots were enumerated. Individual serum

titer from five mice is plotted (in A) and stimulation of T cells (in B) is expressed as mean ±SEM (n=5). Multiple t-tests were applied and statistical significance was determined using Holm-Sidak method (*p* value indicated).

Table 1

Summary of HLA-A mismatches between lung allograft donors and recipients, and clinical features following human lung transplantation.

I.D.	Disease[a]	LTx[b]	HLA-A allele: Donor		HLA-A allele:Recipient		DSA-A[c]	Auto Ab[d]	BOS[e]	AR[f]
P1	CF	1	A2	A33	A3	A68	+	+++	2	2
P2	IPF	1	A3	A33	A2	A24	+	++	3	2
P3	IPF & RA	1	A3	A3	A2	A24	+/−	+	0-p	1
P4	COPD	1	A2	A26	A3	A11	+/−	+/−	Neg	2
P5	A1AD	1	A2	A2	A25	A32	+	++	1	1
P6	IPF	1	A2	A3	A24	A25	+/−	++	1	2
P7	IPF	1	A2	A30	A66	A69	+/−	+	Neg	1
P8	COPD	1	A3	A68	A43	A68	+/−	++	Neg	2
P9	CF	1	A2	A29	A11	A30	+	+/−	0-p	1
P10	CTD	1	A2	A34	A69	A80	+	+++	2	2
P11	CF	1	A11	A33	A3	A24	+	++	Neg	2
P12	COPD	1	A11	A33	A2	A3	+	++	0	1
P13	IPF	1	A30	A32	A3	A68	+	+++	1	3
P14	CF	1	A24	A66	A2	A31	+/−	+	0-p	2
P15	PAH	1	A69	A80	A3	A26	+	+/−	Neg	2

[a] CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis; RA, rheumatoid arthritis; COPD, chronic obstructive pulmonary disease; A1AD, alpha 1 anti-trypsin deficiency; CTD, connective tissue disease; PAH, pulmonary arterial hypertension

[b] 1, primary transplantation (bilateral); 2, re-transplantation (bilateral)

[c] DSA determination performed by LABScreen Single Antigen assay (One Lambda Inc): +, persistent/ recurrent DSA; +/−, resolved DSA; −, DSA negative

[d] Ab to Collagen V or Kα1Tubulin measured by ELISA: +++, strong positive; ++, moderate positive; +, weak positive; −, negative

[e] Bronchiolitis Obliterans Syndrome (BOS) grade at clinical diagnosis; Neg, negative for BOS

[f] Grade of acute rejection (AR)

Table 2

Frequency of cells observed in clinical BAL samples from human LTx recipients.

Cell type	Frequency (% total ± SD)
Bronchoalveolar lavage cells	100 ± 0
Large and granular BAL cells	25.86 ± 8.51
Autofluorescent cells	23.28 ± 7.43
Leukocytes	20.49 ± 4.31
MHC class II high myelocytes (HLA-DR^{high} and CD11b^{+})	15.21 ± 2.94
Mannose receptor (CD169)$^{+}$ and sialoadhesin (CD206)$^{+}$ cells	9.34 ± 1.56
Scavenger receptor (CD163)$^{+}$ cells	7.22 ± 0.45
Alveolar macrophages	6.87 ± 0.34