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Tolerance of Lung Allografts Achieved in Nonhuman Primates via Mixed Hematopoietic Chimerism

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Abstract

While the induction of transient mixed chimerism has tolerized MHC-mismatched renal grafts in nonhuman primates and patients, this approach has not been successful for more immunogenic organs. Here, we describe a modified delayed-tolerance-induction protocol resulting in three out of four monkeys achieving long-term lung allograft survival without ongoing immunosuppression. Two of the tolerant monkeys displayed stable mixed lymphoid chimerism, and the other showed transient chimerism. Serial biopsies and post-mortem specimens from the tolerant monkeys revealed no signs of chronic rejection. The tolerant recipients also exhibited T cell unresponsiveness and a lack of alloantibody. This is the first report of durable mixed chimerism and successful tolerance induction of MHC-mismatched lungs in primates.

Introduction

Tolerance of allogeneic organs is regularly achieved in murine species after the induction of *durable* mixed chimerism, principally through thymic deletion of alloreactive T cells (1,2). However, tolerant nonhuman primates (NHPs) and human renal allograft recipients have only shown *transient* chimerism, suggesting that tolerance here relies on the peripheral inactivation of donor-specific T cells (3–8). Until now, long-term acceptance of more immunogenic organs, such as the lung, has not been achieved in primate species.

The difficulty in achieving primate lung graft acceptance is probably twofold. First, high levels of alloreactive memory T cells (often found in NHPs) are known to hinder tolerance induction in both sensitized rodents (9,10) and NHPs (7,11). Second, barrier organs, such as the lung, elaborate a strong inflammatory response to antigen-dependent and antigen-independent triggers, reinforcing the immune response to the allograft.

Recently, we induced renal allograft tolerance in NHPs by donor bone marrow transplantation (BMT) 4 months after the kidney transplant (12,13). This protocol separates the inflammatory events of the solid organ transplant from those of BMT, while still

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addressing the issue of alloreactive T cells. We have termed this general protocol “delayed tolerance induction,” which is clinically applicable to both deceased and living donor transplantation (14). In the present study, four monkeys received MHC-disparate lungs on a delayed-tolerance-induction protocol; and three out of four animals exhibited long-term graft survival (>400 days), without signs of chronic rejection (CR), off of immunosuppression. This represents the first report of successful tolerance induction in primate lung transplantation.

Materials and Methods

Experimental design

Six cynomolgus monkeys received lung transplants (LTx) from MHC-mismatched donors. To prevent acute rejection, all monkeys were treated with tacrolimus (FK506), mycophenolate mofetil (MMF) and methylprednisone (MP), along with equine-derived anti-thymocyte globulin (ATG) (ATGAM; Pharmacia and Upjohn, Kalamazoo, MI; 50 mg/kg/day IV $\times$ 3 days) induction. Four monkeys (M2411, M4711, M5110, and M5410) also received anti-IL-6R mAb (tocilizumab; Chugai Pharmaceutical Co.; Tokyo, Japan; 10 mg/kg IV on post-LTx Days 0, 7, 14, and 21).

Four months later, each monkey underwent BMT, as described below. Two monkeys (M5110 and 5410) received two doses of anti-CD8 mAb (cM-T807, Centocor, Inc., Horsham, PA; 5 mg/kg IV on the day prior to and 2 days after BMT) and developed posttransplant lymphoproliferative disease (PTLD) and were removed from study—a phenomenon previously observed in NHP-kidney transplantation (15). Subsequently, we altered our protocol to include a course of anti-IL-6R mAb treatment post-BMT (10 mg/kg IV on post-BMT Days 0, 7, 14, and 21), eliminating the second dose of the anti-CD8 mAb. On this modified protocol, none of the four remaining recipients (M912, M2411, M4012, and M4711) showed signs of PTLD. Following BMT, the recipients were also treated with anti-CD154 mAb (h5C8; 20 mg/kg IV on post-BMT Days 0 and 2, and 10 mg/kg on post-BMT Days 5, 7, 9, and 12). Cyclosporine A (15 mg/kg IM initially, then tapering to 5 mg/kg by post-BMT Day 7) was provided for 28 days following BMT. No further immunosuppression was given. Figure 1 depicts the overall protocol.

Animals

Cynomolgus monkeys (*Macaca fascicularis*) weighing 4–7 kg were used (Charles River Primates, Wilmington, MA). Transplant pairs were selected for compatible ABO blood types. Experiments were performed in accordance with NIH’s *Guide for the Care and Use of Laboratory Animals* and approved by the Massachusetts General Hospital IACUC.

Cynomolgus MHC genotyping

Genomic DNA was prepared from peripheral blood mononuclear cells (PBMC) and splenocytes. Panels of 17 microsatellite loci spanning ~5Mb of the MHC were amplified from the genomic DNA with fluorescent-labeled PCR primers, and fragment size analysis was performed. The microsatellite haplotypes were converted to MHC genotypes based on

our previous cloning and sequencing work (16,17). The MHC haplotypes are presented in Figure 2.

Lung transplantation and bone marrow procurement

Left orthotopic lung transplantation was performed in a manner identical to clinical lung transplantation, as previously described (18).

After euthanasia, the donor vertebrae were fragmented and incubated in RPMI with 1% DNase (1 h at room temperature). Bone marrow cells were extracted from the lysate and re-suspended in cryopreservation media containing 58% pentastarch, 10% dimethylsulfoxide and 32% human albumin. The cells were frozen in liquid nitrogen.

Nonmyeloablative conditioning and bone marrow transplantation (BMT)

Four months after lung transplantation, monkeys were conditioned with total body irradiation (TBI) (1.5 Gy 6 and 5 days prior to BMT), local thymic irradiation (TI) (7 Gy on the day prior to BMT) and ATG (50 mg/kg/day IV $\times$ 3 ending on the day of BMT). Donor bone marrow cells (3×10^8 mononuclear cells/kg IV) were infused as previously described (3,12). Ketorolac (3 mg, IM) was administered to prevent thrombosis on the day of BMT.

Flow cytometric analyses, detection of chimerism and cell sorting

PBMCs, peripheral lymph nodes, spleen and bone marrow cells were labeled with a combination of the following mAbs: CD3 PerCP-Cy5.5 (SP 34-2), CD4 PerCP-Cy5.5 (L200), CD8 PerCP (SK1), CD8 APC (SK1), CD95 FITC (DX2), CD95 APC (DX2), and CD28 PE (CD28.2) (BD Pharmingen, San Jose, CA), CD20 PerCP (2H7) (Biolegend, Inc., San Diego, CA) and Foxp3 APC (236A/E7) (eBioscience, Inc., San Diego, CA). Lymphocytes from the animals treated with anti-CD8 mAb were stained with anti-CD8 PE mAb (DK25, Dako, Inc., Carpinteria, CA). For chimerism analyses, we used an antibody that reacts specifically with certain MHC class I antigens (H38, One Lambda, Inc., Canoga Park, CA). The transplant pairs were chosen based on their differential reactivity to this mAb. The presence of CD31 and CD45RA on T cells was detected using clones WM59 and 5H9, respectively (BD Pharmingen). The fluorescence of the stained samples was analyzed using BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) and FlowJo (TreeStar, Inc., Ashland, OR) software. For assessing memory cell function, fresh PBMCs were gated on lymphocytes and sorted into CD16⁻ CD95⁻ CD28⁺ naïve and CD16⁻ CD95⁺ CD28^{low/high} memory populations using a FACSVantage cell sorter (BD Biosciences). The purity of sorted cells was consistently >95%, as previously described (19).

Measurement of T cell-mediated alloresponses by ELISPOT

ELISPOT plates (Millipore, Bedford, MA) were precoated with 5 μ g/mL of capture antibodies against gamma-IFN or IL-17 (Mabtech, Nacka Strand, Sweden) in phosphate-buffered saline (PBS) and stored overnight at 4°C. The responding cells separated from fresh PBMCs were co-cultured with an equal number of irradiated donor or autologous PBMCs as stimulating cells (1.5×10^5 cells/well), or unstimulated in medium alone, or with phytohemagglutinin at 1 μ g/mL (Sigma-Aldrich Corp, St. Louis, MO). After 44 h incubation at 37°C, the plates were washed and biotinylated detection antibodies (Mabtech) were added

for 2 h at room temperature. After five washes with PBS, streptavidin–horseradish-peroxidase conjugate in PBS BSA 0.5% (Dako, Glostrup, Denmark) was added for 1 h at room temperature, followed by five washes. Finally, 100 μ L/well of 3,3',5,5'-tetramethylbenzidine liquid substrate (Sigma–Aldrich) was added and incubated for 15 min in the dark. The resulting spots were counted with an ELISPOT image analyzer (CTL, Inc., Cleveland, OH), as previously described (7,19,20).

Detection of donor-reactive antibodies

The detection of donor-specific alloantibodies was performed using cytofluorometry, as previously described (20).

Skin grafting

Full-thickness skin from donor and third-party animals was recovered and cryopreserved in freezing media (60% RPMI, 30% fetal calf serum, 10% dimethylsulfoxide). Skin was thawed at room temperature immediately before grafting. After preparation of a full-thickness bed on the recipient's back, the graft was placed using with interrupted 4-0 monofilament sutures. The grafts were assessed visually every week and biopsied every 4 weeks.

Histological analyses

Lung biopsies (obtained by limited thoracotomy) and necropsy specimens were fixed in formalin and stained with hematoxylin and eosin (H & E), Masson's trichrome and Verhoeff's elastic stains. Polyclonal anti-C4d (Biomedica, Vienna, Austria) was used to detect C4d in paraffin-embedded tissue. Examination of the tissue was performed by transplant pathologists (R.N.S. and R.B.C). Acute cellular rejection (ACR) was scored using the grading system promulgated by the International Society of Heart and Lung Transplantation (ISHLT), ranging from 0 (no rejection) to 4 (high-grade rejection).

Results

Leukocyte reconstitution, donor chimerism and lung transplant rejection

In all monkeys, leukocyte reconstitution was characterized by an early and transient expansion of CD4⁺ CD25^{high} FOXP3⁺ regulatory T cells and a massive homeostatic expansion of T cells (mostly CD8⁺) displaying a memory phenotype, as previously reported in monkeys treated with similar protocols (8). M4711 displayed no evidence of donor chimerism (Figure 3) and developed ACR along with severe obliterative bronchiolitis (OB) on Day 176 post-BMT (Figure 4 and Table 1). In contrast, tolerant animal M912 exhibited low and transient multi-lineage donor chimerism detectable until Day 75 post-BMT. This monkey did not develop ACR (graft survival >463 days), but several biopsies prior to BMT revealed mild lymphocytic bronchiolitis, which did not progress to OB. M2411 and M4012 displayed durable mixed chimerism (813 days and 299 days post-BMT, respectively) (Figure 3) together with long-term graft survival. Mild bronchiolitis, but no OB, was seen on two biopsies (Day 50 and Day 337 post-LTx) in M4012. Mild bronchiolitis and some possible focal OB were seen at multiple time points in M2411. However, terminal histological examination of tissue from these three recipients (collected at the time of

euthanasia, 299–813 days post-BMT) revealed no signs of inflammation, ACR, or OB (Table 1, Figure 4).

Phenotype of donor leukocytes in stable mixed chimeric monkeys

Next, we analyzed the nature of the donor leukocytes in the stable chimeras, M2411 and M4012. We observed that the levels of myeloid chimerism were initially substantial and prolonged (>120 days), but ultimately became undetectable 100–200 days after BMT (Figure 3). However, these monkeys displayed a continuous presence of donor lymphocytes, whose frequencies peaked approximately 100 days after BMT. Then, donor lymphocytes progressively declined to 2–5% and remained stable at this level. Phenotypic analyses revealed three distinct lymphocyte subsets: (1) classical CD4⁺ T lymphocytes, expressing CD3 and TCR surface markers, but not FOXP3; (2) classical CD8⁺ T lymphocytes, again expressing CD3 and TCR surface markers, but not FOXP3; and (3) a population of B cells, expressing MHC class II (DR), CD20 and IgM and IgG B cell receptors (BCR). Interestingly, these donor-derived B lymphocytes displayed an unconventional phenotype, universally expressing CD8—a marker traditionally confined to T cells, NK cells and some dendritic cells. Finally, the proportion of each cell subset among donor lymphocytes varied considerably overtime. Initially, more than 90% chimeric cells were comprised of B cells. Subsequently, the percentage of donor B cells gradually declined, becoming virtually undetectable by Day 200 following BMT. Conversely, the percentage of T lymphocytes increased steadily until Day 200, and was comprised of comparable proportions of CD4⁺ and CD8⁺ T cells (Figure 5).

Influence of donor/recipient MHC gene matching

As shown in Figure 2 and Table 1, M4711 (which rejected without chimerism) had been transplanted with a fully MHC-mismatched lung. However, M912, which developed transient chimerism, had been transplanted with some degree of MHC-matching (two out of four matches at class I, and two out of four matches at class II). The two other tolerant monkeys (M2411 and M4012), which developed durable mixed chimerism received grafts that were MHC-haploidentical. This suggests that the successful induction of mixed chimerism and tolerance in this model required some degree of MHC-matching.

Tolerant monkeys display *in vitro* and *in vivo* donor-specific unresponsiveness

Next, we investigated T and B cell responses in each transplant recipient by testing for the presence of donor-specific antibodies prior to lung transplantation and serially for the duration of the experiment. None of the recipients had alloantibody prior to lung transplantation. However, M4711 (which rejected without chimerism) developed donor-specific antibodies following lung transplantation, which rose considerably after donor BMT (Figure 6). Presumably, sensitization to donor antigen prior to BMT prevented the development of chimerism, ultimately leading to graft rejection.

In another set of experiments, we measured donor-specific T cell pro-inflammatory immunity using an ELISPOT designed to determine the frequency of directly alloreactive memory T cells producing gamma-IFN or IL17, as described elsewhere (20). Prior to transplantation, a high frequency of activated pro-inflammatory T cells secreting gamma-

IFN were detected with allogeneic donor and third-party antigen-presenting cells (APCs) (Figure 7), but not with autologous APCs or media (<8 spots/million T cells), as is regularly observed in primary mixed-lymphocyte reactions (21). This response was primarily mediated by CD8⁺ effector-memory T cells, as previously reported (19).

Interestingly, we also detected modest, but significant, frequencies of IL-17-producing memory T cells prior to transplantation (Figure 7), an observation that has not been previously documented in primates. Posttransplantation, monkeys M912 and M2411 clearly demonstrated a lack of anti-donor (but not anti-third-party) reactivity by T_h1/T_c1 (gamma-IFN) and T_h17 (IL-17) cells throughout the experiment. The T cell response to phytohemagglutinin was similar to that observed pretransplantation (data not shown). Therefore, all monkeys exhibiting long-term survival displayed donor-specific T cell unresponsiveness and a lack of donor-reactive antibodies.

These observations are further corroborated by our finding that M2411 accepted a frozen skin allograft from its donor for over 134 days, while acutely rejected a third-party skin graft (Figure 8).

Discussion

In the present study, transient or stable mixed hematopoietic chimerism was achieved in three monkeys, which accepted lung allografts with no CR for extended periods of time after the withdrawal of immunosuppression. *In vitro* assays revealed that these monkeys were tolerant of donor antigens, displayed donor-specific T cell unresponsiveness, and never developed alloantibody. While transient chimerism was sufficient to ensure long-term survival of lung allografts without CR in one recipient, it remains to be determined whether its state of unresponsiveness was as robust as that achieved in the stable chimeras.

Previous studies have demonstrated that transient mixed chimerism is sufficient to result in indefinite survival of kidney allografts in NHPs and patients (3–5). However, some of these monkeys ultimately mounted an alloantibody response and developed chronic humoral rejection of their grafted kidneys (8), often developing an indirect T cell alloresponse traditionally associated with antibody production and CR (22–25). These observations suggested that transient chimerism is sufficient to prevent ACR resulting from directly activated T cells, but may not prevent some T cells from being activated indirectly. In contrast, none of the chimeric monkeys tested in this study developed alloantibodies and CR, a result suggesting that no indirect alloresponses were induced. Further studies exploring the relationships among chimerism, indirect alloresponses and CR are needed to confirm this hypothesis.

Stable mixed chimerism is regularly achieved through nonmyeloablative conditioning, leukocyte costimulation blockade and allogeneic BMT in rodents, but not in primates. It has become increasingly clear that this difference relates in part to the higher frequency of alloreactive memory T cells present prior to transplantation in NHPs (7,26). Indeed, mice sensitized to alloantigens or adoptively transferred with alloreactive memory T cells do not develop durable hematopoietic chimerism and demonstrate resistance to tolerance induction

(26–28). In primates, alloreactive memory T cells are generated through infections and immunizations (heterologous immunity) or through direct exposure to allogeneic MHC antigens. We have previously reported that successful tolerance of NHP kidney allografts is dependent upon lower frequencies of preexisting donor-specific memory T cells in the recipients. It is important to note that in the present study both monkeys which developed stable donor chimerism displayed substantial frequencies of donor-reactive memory T cells pretransplantation (>1000 /million memory T cells for M2411), and that our protocol was sufficient to overcome this barrier.

The stable donor chimerism obtained in both M2411 and M4012 was confined to lymphoid cells. Initially, the donor lymphocytes found in both chimeric monkeys were essentially comprised of B cells ($CD20^+$, MHC class II $^+$, and BCR $^+$) displaying an unconventional phenotype characterized by the expression of CD8 surface glycoproteins. Further investigation will be required to determine whether the chimeric B cells identified in our study play any significant role in the induction and/or maintenance of tolerance in this model.

Nonetheless, it is still unclear why two out of four of our lung-transplanted monkeys became stable mixed chimeras, while our kidney-transplanted monkeys have never achieved durable chimerism using a similar protocol (3,7,8). We should note that these two lung recipients were haploidentical, and that MHC-antigen sharing (particularly at the class II locus) can exert a beneficial influence on peripheral T cell regulation and tolerance induction (29). However, the benefit conferred by MHC class II matching is probably not sufficient, in and of itself, to account for stable chimerism in these monkeys, since this has never been observed in monkeys treated similarly and transplanted with haploidentical kidneys (3,7,8). We, therefore, suspect that the inclusion of the anti-IL-6R mAb (known to block inflammation and promote regulatory T cell proliferation) to the conditioning regimen facilitated the establishment of durable mixed chimerism and lung allograft tolerance. Work is in progress to determine the precise contribution of IL-6R blockade to LTx tolerance via mixed chimerism in this NHP model.

This manuscript represents the first report describing both stable mixed hematopoietic chimerism and long-term survival of allogeneic LTx without ongoing immunosuppression in NHPs. LTx tolerance was associated with sustained donor-specific T cell nonresponsiveness, lack of alloantibody and the absence of CR. We anticipate that this protocol, in which tolerance was induced 4 months after lung transplantation, will have clinical applicability to recipients of lungs from both deceased and living donors, and may offer hope to a group of patients whose survival after transplantation currently remains poor.

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Abbreviations

ACR	acute cellular rejection
APC	antigen presenting cell
ATG	anti-thymocyte globulin
BCR	B cell receptor
BMT	bone marrow transplantation
CR	chronic rejection
FK506	tacrolimus
H & E	hematoxylin and eosin
LTx	lung transplant
MMF	mycophenolate mofetil
MP	methylprednisone
NHP	nonhuman primate
OB	obliterative bronchiolitis
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PTLD	posttransplant lymphoproliferative disease
TBI	total body irradiation
TI	thymic irradiation

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Figure 1. Delayed tolerance induction protocol

The sequence and timing of the protocol interventions are depicted relative to the timing of LTx and BMT. The lung allografts were maintained with conventional triple-drug immunosuppression for 4 months, followed by BMT using a nonmyeloablative conditioning regimen. After BMT, the recipients were treated with short courses of anti-CD154 mAb and anti-IL-6 receptor mAb, a single dose of anti-CD8 mAb, along with a 28-day course of CsA. After this, no further immunosuppression was given.

	M2411		M1711		M4012		M5012	
MHC class I A	?	A1*063:02	A1*063:02	A1*031:01	A1*063:01	A1*063:02	A1*063:01	A1*033:01
	?	A2*05:11	A2*05:11	A2*05:04	A2*05:01	A2*05:11	A2*05:01	A2*05:21
	?	A4*01:01	A4*01:01	A5*30:01	A4*01:01	A4*01:01	A4*01:01	A4*01:01
	B*057:02	B*011:01	B*057:02	B*027:02	B*057:02	?	B*057:02	B*036:01:02
	B*064:01	B*075:01	B*064:01	B*088:01	B*064:01	?	B*064:01	B*037:01
MHC class I B	B*104:01:01	B*079:01	B*104:01:01	B*147:01	B*104:01:01	?	B*104:01:01	B*045:01
	B*134:02	B*070:02	B*134:02	B*051:03	B*134:02	?	B*134:02	B*050:04
	B*144:02	B*098:05	B*144:02	B*060:05:01	B*144:02	?	B*144:02	B*060:01
	B*046:01:01	B*165:01	B*046:01:01	B*127:nov:01	B*046:01:01	?	B*046:01:01	B*051:04
	B*060:05:02		B*060:05:02		B*060:05:02	?	B*060:05:02	B*098:04
MHC class II DR	B*131:02		B*131:02		B*131:02	?	B*131:02	B*149:01
	B*152:01N		B*152:01N		B*152:01N		B*152:01N	B*167:01
	DRA*02010101	DRA*0102	DRA*02010101	DRA*0102	DRA*02:01:01:01	DRA*01:02	DRA*02:01:01:01	DRA*01:02
	DRB*W501	DRB1*1002	DRB*W501	DRB4*0101	DRB*W5:01	DRB1*10:02	DRB*W5:01	DRB4*01:01
	DRB*W2101		DRB*W2101	DRB5*030101	DRB*W21:01		DRB*W21:01	DRB5*03:01:01
MHC class II OQ	DQA1*2403	DQA1*0503	DQA1*2403	DQA1*0107	DQA1*24:03	DQA1*05:03	DQA1*24:03	DQA1*01:07
	DQB1*1801	DQB1*1601	DQB1*1801	DQB1*0608	DQB1*18:01	DQB1*16:01	DQB1*18:01	DQB1*06:08
MHC class II DP	DPA1*0702	DPA1*0202	DPA1*0702	DPA1*0401	DPA1*07:02	DPA1*02:02	DPA1*07:02	DPA1*04:01
	DPB1*42	DPB1*29	DPB1*42	DPB1*12	DPB1*19:03	DPB1*09:02	DPB1*19:03	DPB1*03:03

	M912		M1012		M4711		M3012	
MHC class I A	A1*063:01	A1*063:02	A1*063:01	A1*031:01	A1*032:01	A1*060:05	A1*063:01	A1*031:01
	A2*05:01	A2*05:11	A2*05:01	A2*05:04	A1*047:01	A5*30:01	A2*05:01	A2*05:04
	A4*01:01	A4*01:01	A4*01:01	A5*30:01	A4*01:09		A4*01:01	A5*30:01
	B*019:03	B*011:01	B*011:01	B*027:02	B*033:01	B*044:04	B*057:02	B*036:01:02
	B*079:03	B*075:01	B*075:01	B*088:01	B*060:04	B*072:02	B*064:01	B*037:01
MHC class I B	B*148:01:01	B*079:01	B*079:01	B*147:01	B*095:01	B*164:02	B*104:01:01	B*045:01
	B*150:01:01				B*151:01		B*134:02	B*050:04
							B*144:02	B*060:01
	B*098:01	B*070:02	B*070:02	B*051:03	B*045:03	B*060:06	B*046:01:01	B*051:04
	B*109:04	B*098:05	B*098:05	B*060:05:01	B*046:09	B*166:01	B*060:05:02	B*098:04
MHC class II DR	B11L*01:nov:01	B*165:01	B*165:01	B*127:nov:01	B*098:06	B11L*01:05	B*131:02	B*149:01
					B11L*01:04		B*152:01N	B*167:01
	DRA*0102	DRA*0102	DRA*0102	DRA*0102	DRA*0102	?	DRA*02:01:01:01	DRA*01:01
	DRB1*1002	DRB1*1002	DRB1*1002	DRB4*0101	DRB1*0402	DRB1*0302	DRB*W5:01	DRB5*03:01:01
				DRB5*030101	DRB*W401	DRB*W103	DRB*W21:01	
MHC class II OQ	DQA1*0503	DQA1*0503	DQA1*0503	DQA1*0107	DQA1*0108	DRB*W3601	DQA1*24:03	DQA1*01:06
	DQB1*1601	DQB1*1601	DQB1*1601	DQB1*0608	DQB1*060102	?	DQB1*18:01	DQB1*06:11
MHC class II DP	DPA1*0202	DPA1*0202	DPA1*0202	DPA1*0401	DPA1*0203	?	DPA1*07:02	DPA1*02:03
	DPB1*29	DPB1*29	DPB1*29	DPB1*12	DPB1*21	?	DPB1*19:03	DPB1*04:01

Figure 2. MHC haplotypes of donor–recipient pairs

The MHC class I (A and B loci) and II (DP, DO, and DR loci) genes expressed by each of the recipient monkey (M2411, M4012, M912, and M4711) and its respective donor are shown and color-coded to highlight allelic differences.

Figure 3. Evaluation of donor hematopoietic chimerism in transplanted monkeys

The frequencies of different donor-derived leukocyte subsets, including lymphocytes, monocytes and granulocytes were assessed in each lung-transplanted monkey, at different time points after bone marrow transplant (BMT). Fresh leukocytes in the host peripheral blood were stained with mAb against HLA class I Bw6 expressed on donor cells, but not on recipient cells. The frequencies of Bw6 positive cells were measured by FACS. Dead cells were excluded by propidium iodide staining. M2411 and M4012 exhibited high levels (>20%) of durable lymphoid chimerism, as well as long-lasting myeloid chimerism. M912 showed multilineage chimerism, although it was transient (75 days). No donor chimerism was developed in M4711.

Figure 4. Pathology and chest radiograph of lung recipients

Shown are photomicrographs (H & E staining) of lung biopsies performed at the time of euthanasia and chest radiographs obtained at indicated time points from each monkey. The chest radiographs of M2411, M912, M4012 displayed well-aerated lung allografts in the left thoracic space. M4711 showed diffuse infiltration of the transplanted lung on Day 294 post-lung Tx (Day 176 post-BMT). No signs of rejection were seen in the lung graft biopsy of M2411, M912, and M4012. The lung allograft in M4711 exhibited findings of severe acute cellular rejection, vasculitis, and OB on Day 306 post-lung Tx (Day 188 post-BMT). BMT, bone marrow transplantation; H & E, hematoxylin and eosin; OB, obliterative bronchiolitis; Tx, transplant.

Figure 5. Nature of donor lymphocytes persisting in stable chimeras

The phenotype and frequency of different donor lymphocyte subsets were evaluated by FACS in the peripheral blood of the lung transplant recipients exhibiting durable chimerism after donor bone marrow cell infusion (M4012 and M 2411). The results are expressed as percentages of T cells, B cells, CD8⁺ B cells and recent thymic emigrants (CD31⁺CD45RA⁺), among donor lymphocytes.

Figure 6. Detection of anti-donor antibodies in transplanted monkeys

Serum samples from each transplanted monkey were collected serially throughout the experiment. The presence of antidonor antibodies in the sera (1/20 dilution) was assessed by FACS as described in the Materials and Methods section and is expressed as mean channel fluorescence (MFI). Three monkeys (M2411, M4012, M912; lungs accepted) had no significant levels of allospecific antibodies throughout the experimental periods, whereas one animal (M4711; lung rejected) developed anti-donor antibody after lung transplantation and subsequent BMT. BMT, bone marrow transplant.

Figure 7. Donor-specific T cell pro-inflammatory immunity

ELISPOT data is presented for M4012, M2411, and M912 showing the frequencies of memory T cells (among 10^6 peripheral blood T cells) producing IFN-gamma or IL-17 after *in vitro* culture with donor or third-party irradiated antigen presenting cells (PBMCs) (responder/stimulator ratio was 1:1) for 48 h (mixed lymphocyte reaction). The numbers of spots recorded with autologous APCs or media were <8 spots/million T cells.

134 days after skin grafting

Figure 8. Acceptance of donor skin

Full-thickness skins from a lung donor and an allogeneic third-party were grafted on Day 679 post-BMT in M2411. Photograph (left panel) and photomicrograph (right panel; H & E) were obtained 134 days after grafting. Donor skin was elastic and displayed intact appearance all the time through the observational course while the third-party skin rejected in 20 days. Histology of the donor skin showed intact epidermis in a well-preserved skin

architecture. Scale bar, 100 μ m. BMT, bone marrow transplantation; H & E, hematoxylin and eosin.

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Table 1
Summary of results obtained in lung-transplanted monkeys treated with our delayed protocol

Recipient	Donor	MHC mismatch ⁽¹⁾		Mixed chimerism	ACR at last biopsy	Chronic rejection	Allo-antibody	Graft survival
		MHC I	MHC II					
M4012	M5012	2/4	2/4 Haploidentical	Permanent	ACR 0 Day 610 Post-LTx	None	None	299 days post-BMT (euthanized with no signs of rejection)
M2411	M1711	2/4	2/4 Haploidentical	Permanent	ACR 0 Day 939 Post-LTx	None	None	813 days post-BMT (euthanized with no signs of rejection)
M912	M1012	2/4	2/4	Transient until Day 75 post-BMT	ACR 0 Post-mortem	None	None	464 days post-BMT (euthanized with no signs of rejection)
M4711	M3012	4/4	4/4	None	ACR 3 Post-mortem	Severe OB	Developed post-lung tx	176 days post-BMT ⁽²⁾

ACR, acute cellular rejection; BMT, bone marrow transplantation; LTx, lung transplant; OB, obliterative bronchiolitis.

¹For the cynomolgus MHC class I, we individually considered the A and B regions on each chromosome in a given recipient/donor pair, assigning a value for degree of MHC class I mismatching ranging from 0 to 4 out of 4. For the cynomolgus MHC class II, we consider DR and DQ as one region, and DP as a second region, again assigning a value for degree of MHC class II mismatching ranging from 0 to 4 out of 4.

²Euthanized 12 days later for ACR.