Characterization of waves of leukocyte recruitment to the lung allograft and the effect of CTLA4-Ig

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MHC-mismatched lung allografts are rapidly rejected by the host immune response. We analyzed cells infiltrating the grafted lung tissue using a collagenase-digestion method. The grafted lung was filled with host-derived leukocytes as early as day 1 post transplantation and the majority of the initial infiltrating cells were granulocytes. This initial influx of granulocytes waned rapidly, followed by a steady increase in lymphocytes, particularly T cells, and then by macrophages. The proportion of CD4^{+} T cells that express CD25 were increased in the graft the majority of which were activated CD4^{+} cells. We applied cytotoxic T-lymphocyte-associated antigen 4 (CTLA4)-Ig treatment in combination with donor-specific blood transfusion to the transplantation of lung allograft, which was significantly prolonged by the treatment. To examine the cellular and molecular basis of the inhibition of the graft rejection, we evaluated number and cytokine mRNA expression of the cells infiltrating in the lung allograft using collagenase-digestion method, although we were unable to detect significant effects of the treatment. Taken together, this study demonstrates that single cell suspensions from cellular infiltrates of lung tissue is useful for phenotypical and functional studies on cells infiltrating lung tissue after graft transplantation.

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Introduction

The success of clinical lung transplantation is poor when compared with other organ transplants due to acute rejection. Acute lung rejection is accompanied by infiltration of leukocytes into the alveolar, perivascular, peribronchial and interstitial spaces of the transplanted lung, which increase in severity with the advancement of the rejection processes[1,2]. One of the commonly used methods to monitor alveolar infiltration of leukocytes is to analyze cells in the bronchoalveolar lavage fluid (BALF)[3]. In contrast, histological analysis was mainly used to study cells infiltrating the perivascular or peribronchial spaces. These *in situ* studies showed infiltration of highly proliferative macrophages into the tissue of lung allografts[2]. Histological study does not, however, necessarily reflect the events that occur in the whole organ of the lung. Unlike histological analysis, flow cytometric analysis using single cell suspensions of infiltrating cells permits quantitative analysis of infiltrating cells.

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)-Ig was designed as a passive blocker of T cell activation by binding to activated APCs therefore preventing intracellular

signaling through CD28 on T cells[4]. Studies showed that the CTLA-4-Ig fusion protein dampens pathogenic T cell responses in autoimmunity and transplantation models and it has already entered into the clinical stage of development[5,6]. Successful treatment of lung allo-transplants with CTLA4-Ig has been reported in a combination of Brown Norway (BN) donor to F344 strain recipient that are incompatible with major histocompatibility antigen complex (MHC) molecule, RT1[7]. In other combinations of donor (BN) and recipient (Lewis), CTLA4-Ig alone had little effect, but in combination with sirolimus, a cell-cycle inhibitor, significant prolongation of graft survival was achieved[8]. Donor-specific blood transfusion (DST) is another effective method to induce inhibition of donor-specific immune responses[9]. The combination of DST and CTLA4-Ig was, however, reported not to be effective in inhibiting parameters of graft rejection in BN to Lewis lung transplantation[10], although the method was effective in long term survival of cardiac allografts[11].

We applied a method of collagenase digestion to analyze the population of cells infiltrating the perivascular or perialveolar lung tissue using flow cytometry in the combination of BN donor to Lewis recipient transplantation. In both BALF and enzyme-digested lung tissue the waves of infiltration of the different types of leukocytes, granulocytes and lymphocytes into the graft were observed after lung transplantation. This method allowed us to study the phenotype and cytokine expression profile of the purified $CD4^+$ and $CD8^+$ T cells that infiltrate into the allograft.

Materials and Methods:

Animals

Brown Norway (BN)($RT1^n$) and Lewis (LEW)($RT1^l$) rats weighing 200 to 300 g were purchased from Charles River Japan, and were maintained in the Laboratory Animal Center for Animal Research at Nagasaki University. Age and sex matched rats were used at 8-12 wks of age. The animal experiments reported herein were approved by the Institutional Anima Care and Use Committee of Nagasaki University and were conducted according to the guidelines for Animal Experimentation, Nagasaki University.

Lung transplantation and DST

Orthotopic transplantation of the left lung was performed in an RT1 incompatible combination from BN to LEW using a cuff technique as described previously[12]. In brief, donor animals were anesthetied, and medial sternotomy was performed to expose the lungs. Recipient animals were then anesthetized, a left thoracotomy was performed, and the left lung was dissected and removed following clamping of the pulmonary artery, vein and left main bronchus. All anastomoses were performed with cuff technique. Rats with acute allograft dysfunction immediately following implantation were judged technical failure and excluded from further study. For DST, single cell suspension of donor spleen cells were filtered through sterile gauze and suspended in PRMI1640. After lysis of RBC, cells were washed three times with PBS and were injected through the tail vein of the recipient rat (4.0×10^7/rat). The rejection of the transplanted lung was judged by radiographic monitoring, and the >80% radiographic opacification was considered to be indicative of the rejection as described[8].

Cell preparation and flow cytometry

BAL was performed by canulation of the trachea with a 14-gauge catheter secured by suture. PBS (9ml) was instilled into the main-stem bronchus and aspirated. After BAL, lungs were resected, avoiding lymph node tissues. Lung tissues were minced with scissors and enzymatically digested in RPMI1640 containing collagenase A (10 mg/ml) at 37°C for 60 min. Cells were filtered through sterile mesh, washed and resuspended in staining media.

The staining reagents used in this study include PE-anti-CD4, FITC-anti-CD8, biotin-anti-CD25, biotin-anti-TCR, biotin-anti-RT1A, streptavidin-APC and FITC-anti-RT1A[a,b,1] (BD Biosciences). 7-amino-actinomycin D was added to exclude dead cells from the analysis. For analysis of foxp3 expression, cells were stained with FITC-CD4, biotin-anti-CD25 plus streptavidin-APC, fixed, permeabilized and stained using a foxp3 staining kit (eBiosciences). Samples were analyzed using FACScan or FACSCanto II (BD Biosciences).

CTLA4-Ig

Complementary DNA for CTLA4-Ig protein was prepared by PCR-amplification of the extracellular domains of mouse CTLA-4 from $CD25^+$ BALB/c spleen cells with primers (5'-ATTCTCGAGCCAGCCATGGCTTGTCTTGG-3' and 5'-CGGGATCCGCGTCAGAATCCGGGCATGGTT-3') that allowed fragments to be digested with *Hin*dIII and *Bam*HI, and ligation in frame to a human Fc IgG2a cDNA fragment encoded in a CDM8 expression vector as described in Current Protocols in Immunology (Hollenbaugh and Aruffo, 2002). Culture supernatant was collected from COS cells

that were transiently transfected with the CTLA4-Ig construct using the DEAE-dextran method as described[13]. After concentration of the supernatant, CTLA4-Ig was purified using a protein A column. Purification of CTLA4-Ig was confirmed by SDS-PAGE on a 12% gel run under reducing conditions and visualized by Coomassie brilliant blue staining.

Real-time PCR

Lung cell suspension was treated with biotin-anti-rat CD4 or biotin-anti-rat CD8 mAb, incubated with streptavidin-IMag, and CD4+ or CD8+ cells were prepared using IMag System, respectively (BD Biosciences). RNA was prepared from cells using Isogen (Nippon gene) and real-time PCR was performed as described previously[14]. Briefly, complementary DNA was generated from 2 g total RNA by using random hexamers and was amplified by real-time PCR using specific primers (Table 1) in the buffer containing SYBER green. The mRNA expression was determined as the ratio of each DNA to G3PDH.

Table 1. Primer sequences used for real-time RT-PCR

name	5' primer	3' primer
IL-2	CCTGTGTTGCACTGACGCTT	TTCCTTTGCAGGGCTTGAAG
IFN-	AACAGTAAAGCAAAAAAGGATGCAT	TGCTGGATCTGTGGGTTGTTC
TNF-	AGCATGATCCGAGATGTGGAA	GGAGGCCCCCCATCTTT
IL-10	AGTGATGCCCCAGGCAGA	GCTTCTCTCCCAATTCA
TGF-	AGGACCTGGGTTGGAAGTGG	AGTTGGCATGGTAGCCCTTG
IL-4	CCAGACGTCCTTACGGCAA	CCCTGGAAGCCCTGCAG
Perforin	GGAAGCAAACGTGCATGTGT	TCGGCTGCAAAATTGGCTAT
granzyme	GCGCTGTGAAGCCTCTCAAT	CCACATAGCACACATCTCCTGG
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Statistics

Data from the different groups were compared using one-way analysis of variance (ANOVA) and examined by Scheffe' multiple comparison test. A two-tailed P value < 0.05 was considered significant. StatView Software, version 5.0 (SAS institute, Inc., Cary, NC) was used for the statistical analysis.

Results

Waves of infiltrating host immune cells into the grafted lung

Orthotopic left lung transplantation was performed in the fully allogeneic BN to LEW rat strain combination. The following day cells were recovered from BALF as well as from the collagenase-digested grafted lung. Cell origin was determined by staining with allele-specific anti-MHC class I antibodies (Fig. 1A). Nearly 90% of the cells recovered from the BALF and enzyme-digested lung were of the host origin. To examine the characteristics of these infiltrating cells in the transplanted lung, the numbers and phenotypes of cells from BALF, lung and spleen were determined
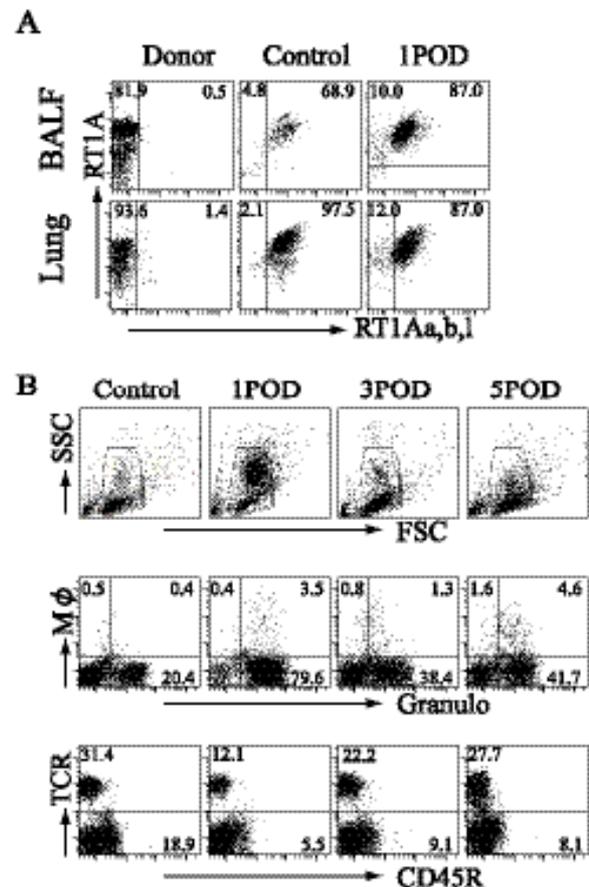
Figure 1. Waves of infiltrating cells into the lung allograft.
(A) Cells were prepared from BALF and collagenase-digested left lung tissue from BN (donor), Lewis (control) and Lewis transplanted with left lung of BN 1 day earlier (1POD). Cells were stained with FITC-anti-RT1Aa,b,l and biotin-anti-RT1A plus streptavidin-PE, and were analyzed using FACScanto II. The numbers indicate the proportions of cells in each quadrant.
(B) Grafted lung tissue from Lewis transplanted with BN lung was isolated 1, 3 and 5 days after transplantation (1,3, 5POD). Control shows the profiles of Lewis left lung. Lungs were digested with collagenase and the single cell suspension was stained with the combinations of FITC-anti-rat granulocyte Ab (clone HIS48) and PE-anti-rat macrophage (clone HIS36), FITC-anti-CD45R and PE-anti-TCR. The gates were set as shown in the profiles of side scatter (SSC) and forward scatter (FSC); macrophage/granulocytes by large gate, and lymphocytes by smaller lymphocyte gate. Numbers indicate the percentage of cells in each quadrant

using flow cytometry on 1, 3, and 5 days after transplantation. The total cell number generally increased in BALF and lung after transplantation, while the spleen cell number was relatively stable (Fig. 2). We next examined the cell types that were recruited during this period using cells re-

covered from the lung and BALF (Fig. 1B, Fig. 2). Flow cytometric analysis of side scatter (SS) vs. forward scatter (FSC) indicated an increase in granular cells on day 1 post transplantation in the lung and BALF, which decreased afterwards on day 3 and 5. Cell surface staining with antibodies clearly indicated that these cells infiltrating the lung tissue on day 1 post transplantation were granulocytes (Fig. 1B). In contrast, the ratio of T cells was reduced on day 1 post transplantation, and the number gradually increased on day 3 and 5 post transplantation. Macrophages (CD45R$^+$ cells) gradually increased and reached a peak level on day 5 post transplantation in the BALF and lung. These changes were not seen in the spleen. Therefore, the transplanted lung was infiltrated by waves of different types of cells, first by a transient increase in granulocytes, second by the gradual increase in T lymphocytes, and by the small but steady increase in macrophages.

We also examined the expression of CD25 and foxp3 in CD4$^+$ T cells, since CD25$^+$foxp3$^+$ T cells are regulatory T cells, while CD25$^+$foxp3$^-$ T cells are activated T cells. In the lung, the proportions of CD25$^+$foxp3$^+$CD4$^+$ T cells increased on day 3 after transplantation when the number of T cells increased in this organ. The proportion of CD25$^+$foxp3$^-$CD4$^+$ cells, however, increased on day 1 post trans plantation (Fig. 3). Therefore, activated T cells were quickly recruited to the lung followed by an increase in the number of regulatory T cells.
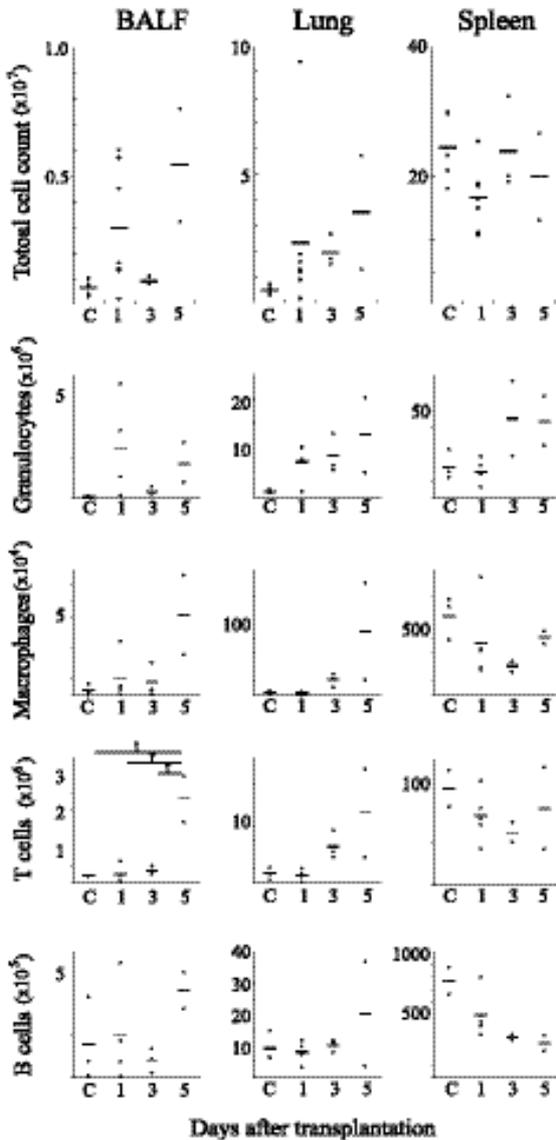
Figure 2. Number of cells recovered from BALF, lung tissue and spleen of the recipient rat.
Cells were recovered from BALF, collagenase-digested lung allograft and spleen from Lewis (control) and from Lewis 1, 3 and 5 days after transplantation (1, 3, 5). Total viable cell numbers were counted under microscopy using trypan blue staining. Cells were stained and analyzed using flow cytometry as shown in Fig. 1. The numbers of granulocytes (HIS48$^+$ cells), macrophages (HIS36$^+$ cells), T cells (TCR$^+$ cells) and B cells (CD45R$^+$ cells) were determined by multiplying the total cell number with the ratio of each cell type obtained from flow cytometric analysis similar to Figure 1B. Small horizontal bar represents the mean value of 2-6 mice.
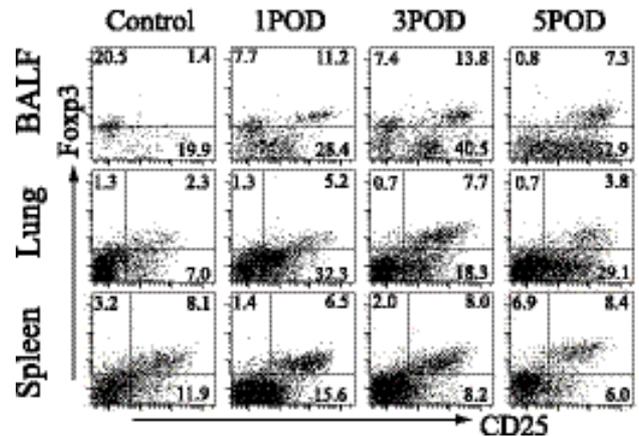
Figure 3. Increase in activated CD4$^+$ T cells in the lung tissue after transplantation.
Grafted lung tissue from Lewis transplanted with BN lung was isolated 1, 3 and 5 days after transplantation (1, 3, 5POD). Control shows the profiles of Lewis left lung. Cells were stained with FITC-anti-CD4 and biotin-anti-CD25 plus streptavidin-APC, permeabilized , and stained with PE-anti-Foxp3 Ab. The profiles of CD25 and Foxp3 expression of CD4$^+$ cells are shown. Numbers indicate the percentage of cells in each quadrant.
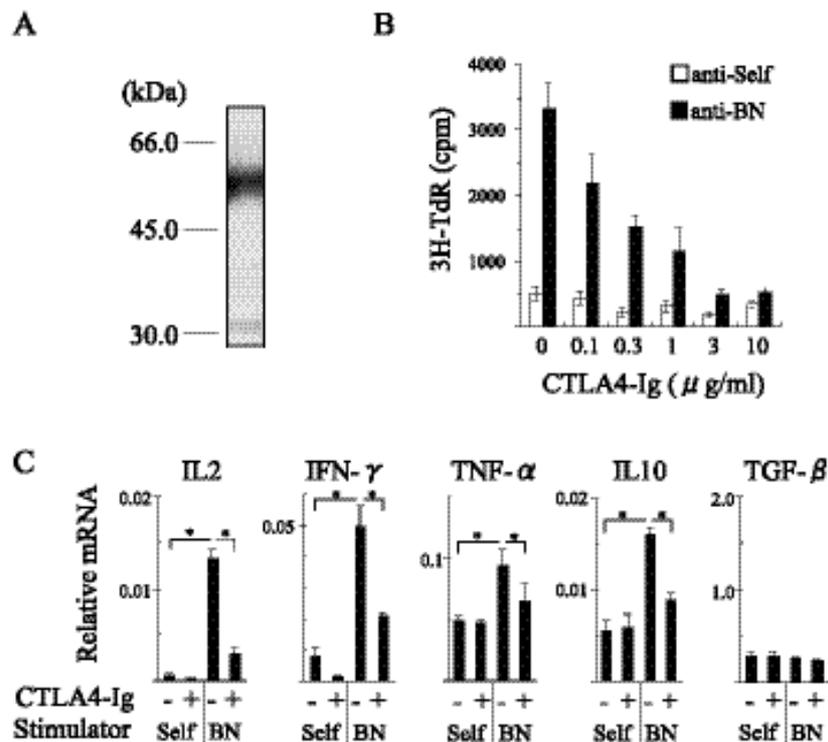
Figure 4. Inhibitory effects of CTLA-4-Ig on CD4^{+} T cell responses *in vitro*.
(A) Purified CTLA4-Ig was subjected to 12% SDS-PAGE under reducing conditons and was visualized by Coomassie brilliant blue staining.
(B) Splenic CD4^{+} T cells (2×10^5) were purified from Lewis and were cultured with mitomycin C-treated spleen cells from syngeneic (anti-self) or BN (anti-BN) rat in the presence of 0-10μg/ml of CTLA4-Ig for 3 days. Proliferation was assed by [^{3}H]-TdR incorporation for 12 hrs at the end of culture.
(C) Lewis CD4^{+} T cells (3×10^6) were cultured with mitomycin C-treated spleen cells (3×10^6) from syngeneic (self) or BN (BN) rat in the presence and absence of CTLA4-Ig (3μg/ml) for 3 days. RNA was extracted and the levels of the indicated cytokine mRNA were determined by real-time PCR. * $p < 0.05$.

Effect of CTLA4-Ig on the host T cell response and allograft rejection

We generated CTLA4-Ig to inhibit lung transplant rejection (Fig. 4A). First, the efficacy of the CTLA4-Ig which we prepared was examined *in vitro*. CTLA4-Ig inhibited MLR in a dose dependent manner (Fig. 4B). Also, CTLA4-Ig in hibited the production of cytokines, IL-2, IFN-γ, TNF-α and IL-10 by CD4^{+} Lewis T cells in response to BN (Fig. 4C). Therefore, we examined the effect of CTLA4-Ig and donor-specific transfusion on the inhibition of acute lung rejection (Fig. 5). CTLA4-Ig or DST alone did not have significant effects on lung allograft survival. However, a combination of the CTLA4-Ig treatment and DST significantly prolonged lung allograft survival from 5.8±0.96 days to 10.7±0.58 days.

Analysis of host cells infiltrating graft following CTLA4-Ig and DST treatment

To examine the cellular basis of the effects of CTLA4-Ig
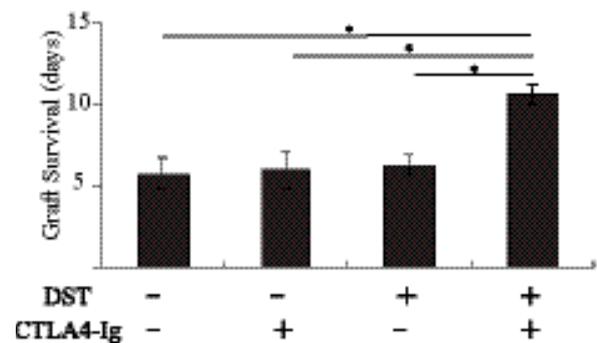
Figure 5. Effect of CTLA-4-Ig and DST treatment on allograft survival. Lewis rats received (DST +) or did not receive (DST -) an *i.v.* injection of BN spleen cells (4×10^7/rat), and then received BN lung allograft. The grafted rats received (CTLA4-Ig +) or did not receive (CTLA4-Ig -) CTLA-4-Ig (500μg/rat) *i.v.* Rejection of the lung allograft was judged by radiographic findings. Data were expressed as mean ± SD. * $p < 0.05$.

and DST treatment, we have analyzed cells in the grafted lung tissue. Three days after transplantation cells were prepared from BALF, lung allograft or spleen and analyzed

using flow cytometry. We were, however, unable to find significant differences in the proportions of macrophages, granulocytes, T and B cells among these three groups of cells (data not shown). We also analyzed the proportions of CD4+CD25+ cells in total CD4+ cells. The CD4+CD25+ cells within the graft 3 days after transplantation were mainly activated CD4+ cells and not regulatory T cells (Fig. 3). We observed a slight reduction of these activated CD4+ cells in the lung tissue of rats treated with a combination of DST and CTLA4-Ig, although the differences were not statisti-

cally significant (Fig. 6). We next examined the cytokine mRNA levels produced in CD4+ and CD8+ cells from these recipient rats treated with and without DST and CTLA4-Ig. This type of analysis was made possible by preparing single cell suspensions of lung-derived cells using the collagenase digestion method. We examined cells from at least 3 different rats in each group. However, the variations between these individual mice in each group were quite large, and we were unable to obtain significant differences in the cytokine mRNA levels between these 4 groups of rats (Fig. 7).
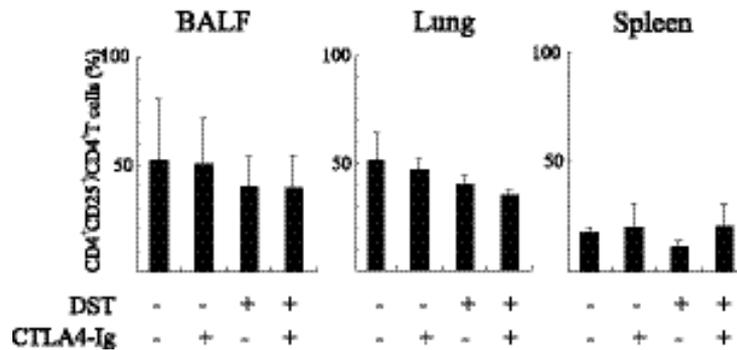
Figure 6. CD4+C25+ T cells in the allograft.
Lewis rats received (+) or did not receive (-) i.v. injection of BN spleen cells (4x10^{7} cells/rat, DST), and received BN lung allograft. The grafted rats received (+) or did not receive (-) CTLA4-Ig (500 μg/rat, CTLA4-Ig) i.v., and were sacrificed on day 3 after transplantation.Cell suspensions were prepared from BALF, lung tissue and spleen and were stained with FITC-anti-CD4, biotin-anti-CD25 plus streptavidin-APC, permeabilized and stained with PE-anti-Foxp3 Ab. The graph represents the proportions of CD4+CD25+ cells within total CD4+ cells as determined by flow cytometric analysis.
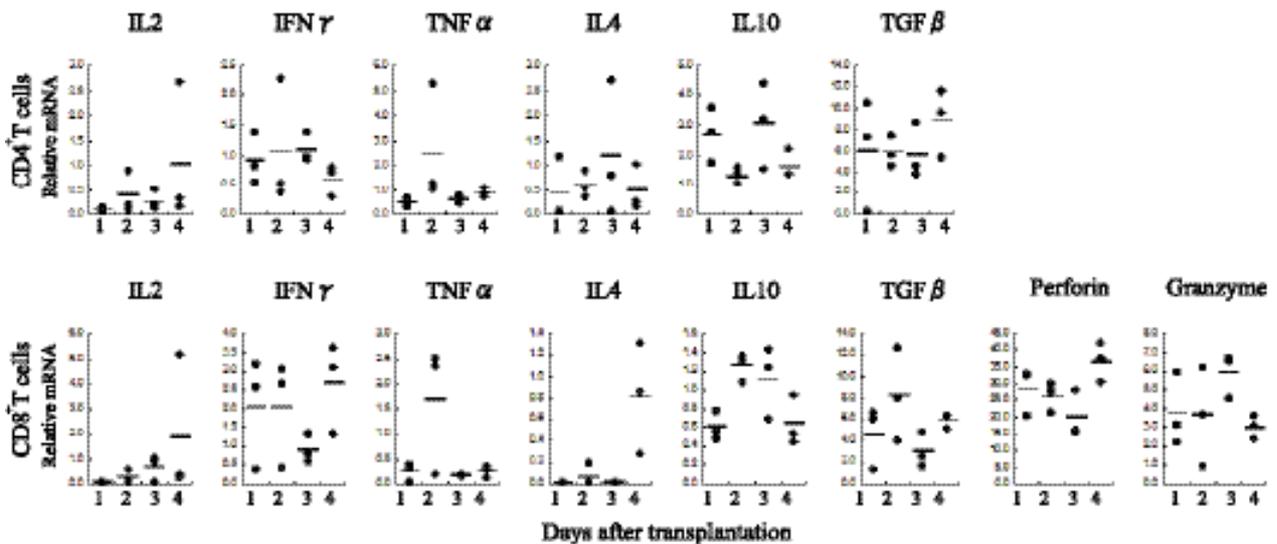
Figure 7. Cytokine mRNA expression in CD4+ and CD8+ cells infiltrating the allograft.
Lewis rats were not treated (1) or treated with CTLA4-Ig (2), DST (3) or both CTLA4-Ig and DST (4), and received BN left lung transplantation. RNA was extracted from CD4+ and CD8+ cells that were purified from the BALF, left lung tissue and spleen of each rat, and real-time RT-PCR analysis of cytokine gene expression was performed.

Discussion

MHC-mismatched lung allografts are rapidly rejected by the host immune response. We have applied a collagenase-digestion method to analyze cells infiltrating grafted lung tissue. Using this method we were able to analyze single cell suspensions of cells infiltrating the perivascular, peribronchial or interstitial spaces of the graft by flow cytometry in addition to BALF. The grafted lung was filled with host-derived leukocytes as early as day 1 post transplantation, and the majority of the initial infiltrating cells were granulocytes. This initial influx of granulocytes in the lung tissue on post-operative day 1 may be due to the ischemia-reperfusion injury that coincides with the increase in TNF levels after lung transplantation[15]. These granulocytes waned rapidly, followed by a steady increase in lymphocytes, particularly T cells, and then by macrophages. The proportion of CD4+ T cells that express CD25 were increased in the graft. The majority of the increase in CD4+CD25+ cells was due to activated CD4+ cells, since the proportions of CD25+foxp3+ cells were not significantly different from those in the spleen.

CTLA4-Ig is a potent immunomodulatory reagent that has approved clinical applications[4,6,16,17]. In combination with additional inhibitory reagents such as anti-CD40 Ab, it can support the long-term acceptance of cardiac, skin or lung allografts in animal models[7,16]. The combination of CTLA4-Ig and DST was also used for the transplantation of cardiac allografts resulting in long-term acceptance[11]. Inhibition of the interaction between CD80/CD86 and CD28 or CTLA4 by CTLA4-Ig results in profound inhibition of T cell immune responses. Recent studies suggested that a complex immunoregulatory network is involved in inhibition of immune responses. In addition to the blockade of co-stimulation, CTLA4-Ig has multiple effects in the regulation of T cell function[6]. CTLA4-Ig regulates tryptophan catabolism in dendritic cells by inducing indoleamine 2,3 dioxygenase (IDO), which degrades tryptophan, resulting inhibition of T cell activation[18]. CTLA4-Ig also has more direct effects on CD4+foxp3+ regulatory cells. Pretreatment of MHC-mismatched rat renal allografts with CTLA4-Ig gene transfer resulted in the infiltration of grafts with foxp3+ T cells with regulatory activity[19]. Another local gene therapy study demonstrated a significant increase in splenic CD4+CD25+ cells with Treg activity in rats bearing pancreaticoduodenal grafts that had been transfected with an adenoviral vector encoding CTLA4-Ig[20]. In contrast, CTLA4-Ig has an inhibitory effect on the development or homeostasis of CD4+CD25+Foxp3+ Treg cells in some models such as the NOD mouse[21]. We have applied CTLA4-Ig treatment in combination with DST since this method was previously shown to be effective in the inhibition of allograft rejection[11]. However, we observed only a slight increase in prolongation of graft survival. This result may have been due to the low dose of CTLA-4-Ig used in the study due to a limited supply of the protein. Therefore, we were unable to observe significant differences in the number and types of cells that infiltrate the lung allograft with and without CTLA4-Ig treatment. Although we also examined mRNA expression of cytokine genes in infiltrating CD4+ and CD8+ T cells, we were unable to observe significant effects of the treatment with CTLA4-Ig and DST. However, our use of collagenase treatment to obtain single cell preparations from lung allografts provided us with a method to examine the types and functions of cells that infiltrate the transplanted lung and will be useful in future studies as well.

Taken together, this study showed the infiltration of waves of donor-derived granulocytes, macrophages and T lymphocytes into the lung allograft. Also, phenotypical and functional studies of single cell suspensions from cellular infiltrates of lung tissue provide us with a useful method for detailed studies on cells infiltrating lung tissue after graft transplantation.

Acknowledgement:

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