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Interferon gamma and T cells inhibit osteogenesis induced by allogeneic mesenchymal stromal cells

Abhijit S. Dighe^{1,a}, Scott Yang^{1,a}, Vedavathi Madhu^{1,a}, Gary Balian^{1,2}, Quanjun Cui^{1,*}, and Orthopaedic Research Laboratories

¹Department of Orthopaedic Surgery, University of Virginia, Charlottesville, Virginia, USA

²Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia, USA

Abstract

The mesenchymal stromal cells (MSCs) are reported to be immunoprivileged and osteogenic. We hypothesized that the use of allogeneic MSCs for bone repair was possible if they displayed an ability to induce similar osteogenesis in syngeneic as well as in allogeneic hosts. To test this hypothesis we used a cloned bone marrow derived cell, termed D1, isolated from Balb/c mice. The D1 cells were subcutaneously injected in syngeneic Balb/c, allogeneic immunocompetent B6, allogeneic T-cell deficient NCr nude and allogeneic B6 Pfp $-/-$ Rag2 $-/-$ mice that lacks matured T-cells and B-cells as well as NK cell cytolytic functions. D1 cells formed ectopic bones only in syngeneic or allogeneic immunocompromised hosts but not in allogeneic B6 hosts. The lack of T-cells alone in allogeneic NCr mice was sufficient to promote osteogenesis in allogeneic environment. We observed a significantly higher number of T-cells, B-cells, macrophages and significantly higher expression of interferon gamma (IFN- γ) in B6 allogeneic implants as compared to the syngeneic implants. These factors correlated with severe inhibition of expression of alkaline phosphatase, osteocalcin and runx2 genes in the implants from B6 mice. Our data suggests that strategies to inhibit T-cells and IFN- γ functions will be useful for bone repair mediated by allogeneic MSCs.

Keywords

bone; allogeneic stem cells; host immune response; fracture repair; osteogenesis

Introduction

Mesenchymal stromal cells (MSCs) are a potential therapeutic option for bone repair as they can readily differentiate into cartilage and bone.¹ It has been reported that MSCs also have the potential to evade the host immune response indicating that allogeneic stem cells can be used for bone repair. MSCs do not cause significant T-cell proliferation when mixed with allogeneic lymphocytes,^{2–4} probably because of the absence of surface expression of major histocompatibility complex (MHC) class II molecules and CD40, CD40L, CD80, CD86 co-stimulatory molecules.^{5–7} MSCs do not constitutively express interleukin-2, a cytokine

*Corresponding author: Quanjun Cui, Department of Orthopaedic Surgery, P.O. Box 800159, University of Virginia, Charlottesville, VA 22908. Phone: 1-434-243-0236, Fax: 1-434-243-0242, qc4q@hscmail.mcc.virginia.edu.

^aThese authors contributed equally to this work.

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important in the activation of T-cells.^{2,8} The release of soluble factors such as Interleukin-10, prostaglandin E-2, indoleamine 2,3-deoxygenase, and transforming growth factor-beta by MSCs was shown to suppress CD4⁺ T-cell differentiation and also promoted a regulatory T-cell type that can suppress the immune response.^{3, 9–11} MSCs also interfered with antigen presenting cell maturation, including dendritic cells, by down-regulating receptors critical for maturation.^{12, 13}

In a study using a critical sized defect in the rabbit ulna, allogeneic MSCs derived from peripheral blood and loaded into a porous calcium phosphate showed considerably improved bone formation at 12 weeks compared to calcium phosphate alone.¹⁴ In another study of bone repair in a critical sized segmental femoral defect in dogs, allogeneic MSCs loaded onto a hollow ceramic cylinder showed significantly more bone formation at sixteen weeks than cell-free implants even without the use of immunosuppression.¹⁵

Although the results of these in-vitro and in vivo studies are promising with regard to the immunoprivileged status of MSCs, some in-vivo studies in MHC mismatched animal models contradict the theory that MSCs can evade the host immune response.^{16, 17} MSCs were found to elicit no lymphocyte proliferation in vitro but evoked significant cellular and humoral responses when implanted in vivo.^{16, 17} Murine allogeneic MSCs were rejected by MHC class I and class II mismatched hosts, and analysis of these allogeneic cell implants revealed increased proportions of host derived CD8⁺ T-cells, NKT-cells and NK cells by comparison to syngeneic controls.¹⁸ Moreover, in a rabbit critical sized bone defect model, rabbit autologous MSCs but not human xenogeneic MSCs enhanced fracture healing.¹⁹ However, no inflammatory response in terms of serum C - reactive protein levels was seen against the human MSCs. Furthermore, the administration of immunosuppressive drugs in vivo significantly improved bone formation induced by MSCs when compared to non-immunosuppressed groups.^{20, 21}

As MHC mismatched allogeneic MSC implantation animal studies have shown evidence for both bone formation as well as rejection, a systematic analysis to determine if MSCs are capable of evading MHC barriers in different mouse immune models and to produce a clinically relevant bone forming response is imperative.

Materials and methods

Cell culture

Mesenchymal stromal cells (D1) were derived from BALB/c mouse bone marrow in our laboratory²². Briefly, a colony of fibroblast-like cells that responded to parathyroid hormone was isolated from bone marrow stromal cells using a cloning ring. From this cloned population two single cell subclones, D1 and D2 were isolated using serial dilution. The D1 cells produced mineralized matrix and were adipogenic in vivo.²² Cryopreserved D1 cells (passage 3) were grown in Dulbecco's Modified Eagle's Medium (Gibco BRL, Gaithersburg, MD., USA) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, VT., USA), 50 µg/ml sodium ascorbate, 100 IU/ml penicillin G, and 100 µg/ml streptomycin in a humidified atmosphere of 5% carbon dioxide at 37°C. D1 cells in PBS were mixed (1:1 volume ratio) with Matrigel (BD Biosciences, NJ, and USA) at 4°C and kept chilled in a syringe until injection. The D1 cells expressed MHC class I molecules K^d and D^d (S-Figure 1).

Mice

8–10 weeks old Balb/c (H-2^d), B6 (H-2^b), NCr nude and B6 Pfp ^{-/-} /Rag2 ^{-/-} mice (Taconic, NY, USA) were housed in the SPF Vivarium at the University of Virginia, which is fully accredited by the American Association for Accreditation of Laboratory Animal

Care. All animal studies were previously approved by, and conducted in accordance with Animal Care and Use Committee oversight. The mice were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Experimental mice received four subcutaneous injections on the dorsum with D1 cells (passage 4, 1×10^6 cells / 0.3 mL) in Matrigel suspension. Anterior implants were injected just before the scapula above the ribs and the posterior implants were injected in the region between the pelvis and the ribs. The animals were allowed to fully recover from anesthesia before returning them to the vivarium. The primary post-procedural analgesic used was Buprenorphine at the concentration of 0.2 mg/kg. The D1 cells were isolated from a Balb/c mouse bone marrow and were used in Balb/c mice, which were genetically identical with D1 cells and also in B6, NCr nude as well as B6 Pfp^{-/-} Rag2^{-/-} mice that were genetically different than D1 cells. NCr nude mice are athymic, and thus lack T-cell function. B6 Pfp^{-/-} Rag2^{-/-} mice lack matured T cells, B cells and perforin dependent NK cells cytolytic functions. Four mice from each group were sacrificed at 1 week, 3 weeks, and 6 weeks and at each time point 4 implants were used for radiography, H and E staining, micro-CT and real time PCR. In addition we used 3 mice (12 implants) in each group that were sacrificed at 36 hours and 1 week for flow cytometry to determine proportions of host immune cells.

Radiographs

At 3 and 6 weeks post injection, radiographs were taken with a Hewlett-Packard Faxitron (Series X-ray System, 43805N) with settings 32.5 kVp and 42 mAs. The implants were surgically removed from the subcutaneous tissue and placed on a Kodak x-ray film. The films were subsequently developed and placed into a GS-800 Calibrated Densitometer (Bio-rad, CA, USA) to convert the radiograph films into digital images. Density measurements of radiographs were performed with the ImageJ software.

Micro Computed Tomography

After mice were sacrificed at 3 and 6 weeks post injection, micro computed tomography (μ CT) scans using a Scanco vivaCT 40 scanner (Scanco Medical AG, Basserdorf, Switzerland) were performed on the mice to evaluate bone volume in the subcutaneous implants. The settings for the scanner were as follows: Voxel size: $38 \mu\text{m}^3$, X-ray tube potential: 55 kVP, integration time: 145 ms. Bone volume analysis was used to quantify the amount of bone formed, and thresholds for bone detection for defined volumes of interest were set at a range 158 to 1000 Hounsfield units.

Histology & Microscopy

For histological analysis, implants retrieved at 1, 3, and 6 weeks post injection were surgically removed, decalcified using 0.25 M EDTA and fixed in 10% neutral buffered formalin, then dehydrated and embedded in paraffin. Six micrometer sections were stained with hematoxylin and eosin and evaluated by light microscopy.

Real Time PCR

Total RNA was extracted from the subcutaneous implants by homogenizing the samples in TissueLyser (Qiagen, CA, USA) for 6 min at 22 Hz in Qiazol solution (Qiagen, CA, USA) with one 5 mm stainless steel bead (Qiagen, CA, USA). The RNA was purified from the samples using the RNeasy lipid tissue mini kit (Qiagen, CA, USA). The yield and purity of the purified RNA was determined by measuring absorbance at 280, 240 and 260 nm. Using random hexamer primers, RNA was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad, CA, USA) to prepare cDNA. The cDNA (112.5 ng total RNA equivalents) was mixed with iQ SYBR Green Supermix (Bio-Rad, CA, USA) and mRNA expression was determined using the iCycler iQTM (Bio-Rad, CA, USA) real-time PCR detection system.

The PCR protocols involved 40 cycles of denaturation at 94 °C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The threshold cycle number (Ct) for each sample was calculated at the point where the fluorescence exceeded the threshold limit. A standard graph of concentration versus Ct value was plotted for each gene of interest using a serially diluted cDNA. The starting relative quantity in each sample was determined using this standard graph and the starting quantity of each gene was divided by the starting quantity of 18S for normalization. The primer sequences used are: 18S Forward (F) 5'-CGGCGACGACCCATTTCGAAC-3', Reverse (R) 5'-GAATCGAACCCTGATTCCCCGTC-3'; Runx2 F 5'-TTATCAAGGGAATAGAGGG-3', R 5'-AGGACAGAGGGAAACAAC-3'; Osterix F 5'-ACCAGGTCCAGGCAACAC-3', R 5'-GCAAAGTCAGATGGGTAAGT-3'; Osteocalcin F 5'-AGGAGGGCAATAAGGTAGT-3', R 5'-CATAGATGCGTTTGTAGGC-3'; Alkaline Phosphatase F 5'-ACGAGATGCCACCAGAGG-3', R 5'-ACGAGATGCCACCAGAGG-3'; IFN- γ F 5'-TCAAGTGGCATAGATGTGGAAGAA-3', R 5'-TGGCTCTGCAGGATTTTCATG-3'; IL-4 F 5'-ACAGGAGAAGGGACGCCAT-3', R 5'-GAAGCCCTACAGACGAGCTCA-3'.

Fluorescence Activated Cell Sorting

Implants (twelve implants for each group) were harvested after 36 hrs and 1 week. All the implants were crushed and digested with collagenase and DNase at 37°C for 2 hours. A homogeneous suspension of cells in PBS was prepared by filtering the homogenates through a nylon mesh of 100 μ m pore size. The cell suspension was washed with PBS and then the cell suspension was incubated with anti mouse CD16/CD32 monoclonal antibody Clone 2.4G2 (BD Biosciences, MD, USA) to block the Fc receptors. 0.1×10^6 live cells, based on trypan blue exclusion, were stained for each reaction using each labeled antibody and at least 10,000 events were counted on FACS calibur with the machine gated for live cells based on forward scatter and side scatter. Percentages of cells expressing that marker were determined out of the total 10,000 events. The cell suspension was stained with monoclonal antibodies (eBioscience, CA, USA): PE labeled anti Sca-1 Clone D7, PE labeled anti CD45R (B220) Clone RA3-6B2, FITC labeled anti CD4 Clone RM4-5, Alexa Fluor 647 labeled anti CD8 Clone 53-6.7, APC labeled anti CD25 Clone PC61.5, PE labeled anti CD49b Clone DX5 and Alexa Fluor 647 labeled anti F4/80 Clone CI:A3-1 (Biolegend, CA, USA) and analyzed using a FACS calibur flow cytometer (BD Biosciences, MD, USA); the data was analyzed using FloJo software (TreeStar, OR, USA).

Statistical Analysis

Statistical analysis of the averages of density from radiographs, bone volume from μ CT, and relative gene expression from real time PCR was performed with SPSS v.17 (IBM, NY, USA), using one-way ANOVA and post-hoc least significant difference analysis between groups. Analysis of the average proportion of cells from flow cytometry data was performed using the independent sample student t-test. Statistical significance level was set at $p < 0.05$.

Results

T-cells inhibit osteogenesis induced by allogeneic stem cells

Implants retrieved from all the mice except B6, appeared radiopaque (Fig. 1, A – E) indicating the formation of bone. The implants obtained from B6 mice were radiolucent and did not form bone. The radiographic density and bone volume of syngeneic Balb/c implants were significantly higher than implants from the allogeneic mice (Fig. 1, E and G) at week 3. The data indicated that the rate of ectopic bone formation was highest in the syngeneic setting at week 3. At 6 weeks, the radiographic density and bone volume of implants in the

B6 group remained significantly lower as compared to that of the syngeneic implants in Balb/c mice ($p=3.12\times 10^{-3}$ and 0.028 respectively). However, bone volumes of Balb/c implants and the implants from immunocompromised allogeneic hosts were comparable at week 6 (Fig. 1, D – G). The data clearly revealed that allogeneic stem cells could induce osteogenesis in absence of immune cells in NCr and B6 Pfp $-/-$ Rag2 $-/-$ mice. The lack of only T-cells ($p=0.05$, 0.05 in comparison with Balb/c and B6 at week 1) in NCr nude mice was sufficient to allow osteogenesis induced by allogeneic stem cells. Using H and E staining we further determined if immunocompetent allogeneic B6 mice inhibited only mineralization phase or osteogenesis in allogeneic mice (Fig. 2). In support of radiography and Micro CT data, a typical bone histology was observed in the implants retrieved from syngeneic control as well as immunocompromised allogeneic hosts. However, all the components representing typical structure – osteocytes, haematopoietic components, bone were completely absent in implants retrieved from B6 mice (Fig. 2). The presence of relatively fewer nuclei in B6 implants also suggested the death of the cells in B6 implants.

Inhibition of expression of osteogenic genes in allogeneic mice

At 1 week, the implants in the allogeneic groups showed inhibition of the alkaline phosphatase (ALP) (B6 $p=0.038$, NCr nude $p=0.034$, B6 Pfp $-/-$ Rag2 $-/-$ $p=0.049$); osteocalcin (OCN) (B6 $p=7.34\times 10^{-5}$, NCr nude $p=7.34\times 10^{-5}$, B6 Pfp $-/-$ Rag2 $-/-$ $p=1.11\times 10^{-4}$) and runx2 (B6 $p=0.024$, NCr nude $p=0.029$) genes compared to the syngeneic Balb/c group (Fig. 3). There was a trend of enhanced osterix expression in Balb/c implants at week 3. However, there was no significant difference in expression of ALP and OCN genes between syngeneic implants and allogeneic implants at week 6.

The macrophages and B cells are recruited in addition to T-cells to allogeneic implants

To confirm our hypothesis that more number of immune cells are recruited towards allogeneic implants we analyzed the implants for the presence of different immune cell populations. At 36 hours, the proportion of F4/80 expressing macrophages was significantly higher in the allogeneic B6 group compared to the syngeneic Balb/c ($p=0.027$) (Fig. 4A). There was a trend (Fig. 4A) ($p=0.71$) towards an increase in the proportion of CD49b (DX5) expressing NK cells in the allogeneic B6 group and an increase ($p=0.05$) in the proportion of CD4 expressing T-cells in the syngeneic Balb/c group.

At 1 week, the increased proportion of CD8⁺ T cells, CD4⁺ T cells, and B220⁺ B cells in the allogeneic B6 group compared to the syngeneic Balb/c group was statistically significant ($p=0.05$, 0.009 and 0.03 respectively) (Fig. 4B). There was a trend ($p=0.16$) towards more F4/80⁺ macrophages in the B6 group compared to the Balb/c group (Fig. 4B).

The MSCs induce T-regulatory cell population

In syngeneic Balb/c mice, the proportion of CD4⁺CD25^{Bright} T-regulatory cells compared to allogeneic B6 mice was higher at all time points (Fig. 4, C – D). At 36 hours CD4⁺CD25^{Bright} population was observed only in Balb/c mice and at 1 week, the percentage of T-regulatory cells in the syngeneic group was 4 fold higher than that in allogeneic group.

T_H1 immune response inhibits osteogenesis in immunocompetent allogeneic hosts

IFN- γ is a signature cytokine for T_H1 immune response whereas IL-4 cytokine represents T_H2 response. At 1 week, a similar inflammatory response was evident within all the implants by virtue of comparably high levels of IFN- γ in all the groups (Fig. 5). However, at 3 and 6 weeks, IFN- γ levels remained significantly higher only in the allogeneic B6 group compared to the syngeneic or immunocompromised allogeneic groups (3 weeks: Balb/c $p=0.034$, NCr nude $p=0.020$, B6 Pfp $-/-$ Rag2 $-/-$ $p=0.022$; 6 weeks: Balb/c

$p=4.3 \times 10^{-5}$, NCr nude $p=2.2 \times 10^{-5}$, B6 Pfp $-/-$ Rag2 $-/-$ $p=2.5 \times 10^{-5}$). At week 1 IL-4 production in the implants retrieved from Balb/c was 30 folds higher (B6 $p=0.048$, NCr nude $p=0.046$) than the implants from B6 and NCr nude mice. At week 6, however, IL-4 expression in Balb/c implants was more ($p=0.03$) as compared to implants from only B6 mice.

Discussion

The purpose of this study was to investigate if MSCs can induce comparable osteogenesis in syngeneic and allogeneic hosts. We also wanted to identify the types of immune cells responsible for inhibition of osteogenesis, if MSCs failed to induce osteogenesis in allogeneic hosts. The populations of immune cells thus identified can then become potential targets to be modulated to allow use of allogeneic MSCs for bone repair.

Interestingly we found that allogeneic MSCs did not form bone in B6 mice (Fig. 1). The clinical trials using allogeneic MSCs for bone repair in patients with osteogenic imperfecta have shown some success^{23–25}, however, the limited number of subjects (3, 6 and 1 patient/s respectively) treated with allogeneic MSCs is not sufficient to ascertain the success of this approach. Out of 123 clinical trials that used MSCs for a wide range of therapeutic applications, 23 were related to bone and cartilage repair.²⁶ However there is insufficient clinical data to support the use of allogeneic stem cells for bone repair based on recent systematic literature review.²⁷ In fact, data from several clinical trials that employed MSCs show that allogeneic stem cells are not immunoprivileged in vivo.²⁸ This raises a question then how allogeneic MSCs enhanced bone repair in rabbits¹⁴ and dogs¹⁵? It is possible that the types of MSCs may determine the host immune response and clearly, more detailed investigation on this subject is required.

It is generally known that NCr nude mice lack T-cells but the innate immune system in these mice is functional. F4/80+ macrophage recruitment towards the implanted cells in NCr nude mice (NCr nude versus Balb/c, $p = 0.032$; NCr nude versus B6, $p = 0.051$) might have delayed onset of osteogenesis in these mice. We assumed that enhanced recruitment of immune cells towards allogeneic implants was responsible for the delayed osteogenesis in allogeneic mice. Inhibition of ALP, OCN, osterix and runx2 genes in T-cells deficient NCr nude and B6 Pfp $-/-$ Rag2 $-/-$ mice suggested that immune cells other than T-cells were responsible for it. We postulate that T-cell mediated killing of D1 cells in B6 mice did not allow enough number of D1 cells to persist till week 6 (S-Figure 2) Therefore no osteogenesis could be initiated in B6 mice at week 6. We believe that inhibition of ALP expression in allogeneic mice was mediated by higher proportion of macrophages as TNF- α and IL-1 produced by macrophages are reported to inhibit osteogenic differentiation of stem cells by inhibiting mRNA expression of ALP.^{29,30} Interestingly, we observed that mRNA expression of runx2 was higher in B6 Pfp $-/-$ Rag2 $-/-$ mice compared to all other groups at week 6. Unlike expression of ALP, OCN and osterix genes, expression of runx2 did not correlate with levels of IFN- γ and IL-4 in any of the groups. The mechanism responsible for this observation remains to be elucidated.

CD4+CD25^{Bright}FoxP3+ T-regulatory cells suppress T-cell response. The data (Fig. 4C–D) indicated that D1 cells modulated host T-cell response by inducing the T-regulatory cells in Balb/c mice. However, B6 implants are most likely to need greater numbers of T-regulatory cells to allow osteogenesis. T-regulatory cells have been shown to suppress effector T-cell proliferation, and decrease immune responses against allogeneic cells.³¹ MSCs recruit the T-regulatory cells to produce an immunosuppressive milieu.^{32,33} It is possible that these T-regulatory cells inhibit T-cell and B-cell response only in syngeneic mice, or that the proportion of T-regulatory cells in allogeneic mice is not sufficient to modulate the adaptive

immune response in allogeneic B6 mice.^{31–33} The weakness of the study was that FACS analysis of host immune cells was not performed in B6 Pfp $-/-$ Rag2 $-/-$ mice, therefore the mechanisms of immune response to implanted cells in B6 Pfp $-/-$ Rag2 $-/-$ mice are not completely understood.

Our data are in agreement with other reports that allogeneic MSCs are unable to induce osteogenesis and that IFN- γ is produced as the host immune response to allogeneic stem cells.^{18, 34} As we observed significantly more number of T cells in B6 implants than in Balb/c implants at week 1 (Fig. 4) and the implants in the T-cells deficient mice did not show increased IFN- γ , we believe that IFN- γ in B6 implants is T-cells derived. A recent report demonstrated that the natural fracture repair occurred more rapidly in Rag1 $-/-$ mice compared to repair in wild type mice.³⁵ The report clearly revealed that T-cells that are absent in Rag1 $-/-$ mice can inhibit bone repair. Interestingly, bone repair induced by syngeneic MSCs also was inhibited by T cells and interferon gamma.³⁶ In their study the authors found that IFN- γ inhibited runx2 pathway and this inhibition could be reversed through local application of aspirin or through systemic infusion of T-regulator cells. In this study we demonstrate inhibition of MSCs induced osteogenesis through similar mechanisms involving T-cells and IFN- γ in allogeneic hosts.

Conclusions

Our data and reports from other groups^{18, 34–36} collectively reveal that allogeneic MSCs can be used for bone repair if recipient T cells and IFN- γ are inhibited through efficient strategies. Our current study demonstrated that the lack of T_H2 response in allogeneic environment correlated with inhibition of osteogenesis. The strategies to enhance IL-4 expression in allogeneic environment might prove beneficial for allogeneic MSCs mediated bone repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Osteogenesis induced by mesenchymal stem cells in syngeneic and allogeneic hosts
 The implants were harvested after 3 and 6 weeks post injection and imaged (A, B). The X-ray images were converted to digital format (C, D) and radiographic density (E) was calculated using the ImageJ program. The harvested implants were analyzed using Micro-CT and the representative images (F) and comparison of bone volumes (G) are shown in the figure.

Figure 2. H and E staining reveals genuine ectopic bone in all the groups except B6 mice at 6 weeks

Six μm thick sections of the harvested implants were stained with hematoxylin and eosin and visualized by light microscopy. While B6 showed the presence of nuclei (N) only; osteocytes (O), haematopoietic components (H) and bone (B) were observed in all the other groups.

Figure 3. Inhibition of expression of osteogenic genes in allogeneic mice

Using gene specific primers, quantitative expression of alkaline phosphates, osteocalcin, osterix and runx2 was determined in real time PCR assays. The values were normalized against 18S rRNA gene expression in all the samples.

Figure 4. The proportions of host derived immune cells in the implants after 36 hours and 1 week

A cell suspension prepared from harvested implants was incubated with monoclonal antibody 2.4G2 to block Fc receptors. The cells were then stained with monoclonal antibodies against Sca-1, CD8, CD4, CD25, F4/80, B220, CD49b and analyzed by flow cytometry after 36 hours (A) and 1 week (B). The percentages of T-regulatory cells after 36 hours (C) and 1 week (D) were determined in implants from B6 and Balb/c mice. * indicates $p < 0.05$.

Figure 5. T_H1 immune response in the allogeneic immunocompetent group and T_H2 immune response in the syngeneic group

cDNA was prepared from mRNA template isolated from the implants. Gene expression of IFN- γ (A) and IL-4 (B) were determined in real time PCR. The values were normalized to 18S rRNA gene expression in all the samples.