Brief Definitive Report

The Pulmonary Alveolar Proteinosis in Granulocyte Macrophage Colony–stimulating Factor/Interleukins 3/5 βc Receptor–deficient Mice Is Reversed by Bone Marrow Transplantation

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Summary

Mice mutant for granulocyte macrophage colony–stimulating factor (GM-CSF) or the common receptor component (βc) for GM-CSF, interleukin (IL)-3, and IL-5 exhibit a lung disorder similar to human pulmonary alveolar proteinosis, a rare disease with congenital, infantile, and adult forms. Bone marrow transplantation and hematopoietic reconstitution of βc mutant mice with wild-type bone marrow reversed the established disease state in the lungs, defining this disease as hematopoietic in nature. It is likely that the disease involves alveolar macrophages, as donor myeloid cell engraftment into the lungs of mutant recipient mice correlated with reverting both the disease and an abnormal macrophage morphology seen in the lungs of affected animals. *Recombination Activating Gene-2* mutant donor bone marrow, which lacks the potential to develop lymphocytes, reversed the pathology in the lungs to the same extent as whole bone marrow. These data establish that certain lung disorders, if of cell-autonomous hematopoietic origin, can be manipulated by bone marrow transplantation.

Pulmonary alveolar proteinosis (PAP) is a rare lung disease where periodic acid Schiff's reaction (PAS)–positive material is progressively accumulated in the alveolar space (1). The intraalveolar material is pulmonary surfactant, a mixture of surfactant proteins (SP-A, B, C, and D) and phospholipids (2). Human PAP is classified into three categories: adult, infantile, and congenital forms. The infantile and congenital forms lead to early respiratory failure, and the prognosis is poor (3). Adult type is not as severe, but can be complicated by opportunistic infection (1).

Gene knockout techniques have provided a number of mouse models that are similar to human diseases. One example is the PAP-like disease in mice that harbor targeted mutation in the GM-CSF ligand (4, 5) or the βc receptor gene (receptor for GM-CSF, IL-3, and IL-5) (6, 6a). The lungs of the βc receptor mutant mice show a progressive accumulation of PAS-positive proteinaceous substance in the alveolar spaces, as well as peribronchovascular lymphocyte infiltration.

The morphology of alveolar macrophages in the lungs of the mutant mice is grossly abnormal. These macrophages are expanded by cytosolic ingested material and appear distended and foamy. It is therefore possible that GM-CSF produced in the lung may be a necessary signal through the βc receptor for the appropriate degradation of surfactant internalized in alveolar macrophages. Alternatively, as type II pneumocytes are also involved in surfactant production and resorption (7), a nonhematopoietic basis to the disease is also possible.

If alveolar macrophages are a primary cause of the lung disease in βc receptor mutant mice because of a cell-autonomous defect, it should be possible to manipulate the lung disease by bone marrow transplantation. It has been shown that alveolar macrophages are derived from bone marrow precursors in rodent systems (8, 9), and also in humans (10). Although steady-state hematopoiesis of βc mutant mice appeared normal, except for reduced levels of eosinophils (6), we sought to explore the relationship between the lung disease and the hematopoietic system by bone marrow transplantation studies. The data presented define that βc-deficient PAP is hematopoietic in nature, and is likely to involve alveolar macrophages.

Materials and Methods

Mice. All animals used in this study were raised and housed in microisolator cages at the DNAX Research Institute animal facility. The production of the βc mutant animals has been described

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previously (6). These animals have routinely tested negative for typical mouse pathogens. Wild-type (WT) and βc mutant donor and recipient animals were derived from littermate-matched offspring and represent an F_3 generation of 129 × C57Bl/6 background. In other identical experiments, the WT animals used as bone marrow donors were of an F_1 (129 × C57Bl/SJL) genetic backgound. These WT F_1 donor cells were haplocongenic at the Ly 5 locus (Ly 5.1 and Ly 5.2), due to the C57Bl/SJL congenic parent, allowing marking of the donor versus recipient cell populations (11). Recombination Activating Gene-2 (*RAG-2*) mutant animals used for donor cells were of 129 genetic background. 129, C57Bl/6, and C57/SJL are matched for H-2^b.

Bone Marrow Transplantation. Recipient mice were irradiated by two doses of 600 rad, 12–16 h apart, from a ^{137}Cs source. After irradiation, animals were injected intravenously with 10^6 bone marrow cells. Marrow cells were flushed out of the femur and depleted of RBCs by osmotic shock. All recipient animals were 2–3-mo old at the time of transfer except for the recipients in Rag-2 transfer experiment where animals were 7-mo old. All donor animals were 2–3-mo old.

Lung Pathologic Evaluation. Lung sections were prepared for pathology and stained with either hematoxylin and eosin or with PAS as previously described (6). After transplantation at the indicated time periods, random step sections from each mouse (minimum of three) were prepared and placed into a histopathological category.

Cell Preparation from Bronchoalveolar Lavage, Immunohistochemistry, and Protein Determination. Animals were killed and lungs were washed with PBS three times by inserting thin tubing attached to a syringe into the trachea. Centrifuged bronchoalveolar lavage (BAL) cells were cytospun and stained with Wright-Giemsa. For immunohistochemistry, lung tissue was fixed with 4% paraformaldehyde in PBS overnight at 4°C, embedded into OCT compound, and cut into 6-μm-thick sections. After blocking endogenous biotin and peroxidase, the samples were treated with 5% mouse serum for 30 min at room temperature, stained with biotinylated anti-Ly 5.2 antibody for 1 h at room temperature, developed using a LSAB kit (DAKO, Glostrup, Denmark), then counterstained with hematoxylin.

For determination of protein in the BAL fluid, the lungs were washed three times with a total volume of 3 ml PBS. BAL fluid was centrifuged to remove cells and the total protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL).

Peripheral Blood Staining. Peripheral blood was prepared by cardiac puncture, and RBCs were lysed by ammonium chloride. Cells were incubated at 4°C with PBS containing 3% mouse serum with FITC-conjugated anti-Ly 5.1 and biotinylated anti-Ly 5.2 antibodies for 30 min, and then with PE-conjugated streptavidin (Becton Dickinson & Co., Mountain View, CA) for 30 min. Cells were washed and analyzed on a FACScan® machine (Becton Dickinson & Co.).

Results and Discussion

Bone Marrow Transplantation. To address the potential link between hematopoiesis and the lung pathology in βc mutant mice, bone marrow from WT animals was transferred into lethally irradiated βc mutant recipient animals in an attempt to cure the PAP. Mice were evaluated at 8 and 12 wk after bone marrow transplantation.

In control experiments, transfer of WT donor cells into WT recipient animals resulted in lung physiology similar to that of unmanipulated WT animals (Fig. 1 *a*). Transfer of βc mutant bone marrow to βc mutant recipient animals resulted in the typical PAP pathology of discreet intraalveolar material in the alveolar spaces (Fig. 1 *b*). However, transfer of WT bone marrow into βc mutant recipient mice resulted in a striking improvement in the lung pathology at 8 and 12 wk after transfer (Fig. 1 *c*). The intraalveolar substance defining PAP was completely absent or only residual, and in clear contrast to the pathology observed in βc mutant animals that received βc mutant bone marrow (compare Fig. 1 *b* to *c*). To quantitate the reversal of PAP, we performed multiple step sections of the lungs of each of the animals in the three transplanted groups described above. A pathological evaluation of each of these tissue sections was done by placing each specimen into a histopathologic category of 1 (normal lung)–5 (severe lung pathology). (The characteristics of each category are described in the legend to Fig. 2.) Fig. 2 shows that the WT animals that had received WT bone marrow were classified as category 1 or 2. βc mutant animals that had received βc mutant bone marrow were classified as category 4 or 5. βc mutant animals that received WT donor bone marrow were classified as categories 2 or 3 at 8 wk after transplant and as category 3 at 12 wk after transplant. 100% of the βc mutant animals that received WT bone marrow completely cleared the intraalveolar material, the defining characteristic of PAP. However, as the βc mutant animals were undergoing the disease state before transplant, residual damage to the lung tissue was detected after clearance of the disease state, as described below.

The histopathologic category of the lung specimens from animals cured of PAP (WT to βc mutant transplanted group) was not identical to the animals of the WT to WT transplanted group. The types of changes accounting for this difference were independent of the characteristics of PAP and consisted of two general findings. Areas of focal alveolar macrophage accumulation (and possibly type II pneumocytes) were detected. The arrows in Fig. 1 *c* show this cellular accumulation visualized by hematoxylin and eosin staining, and the arrows in Fig. 1 *d* show that the focal accumulation of cells contain PAS-positive material. Other changes in the lung where the disease was reversed included occasional areas of collapsed alveolar septa and a limited amount of fibrosis (data not shown). Collectively, the changes in the lung that distinguish WT to βc mutant transplanted groups from WT to WT transplanted groups are distinct from the progressive pathology of PAP, and may be indicative of reparative processes that accompany the reversal of PAP, as well as the remains of direct damage caused to the lung tissue during the disease state.

Protein Accumulation Was Reversed in WT to βc Mutant Transplanted Animals. To independently verify that the disease was reversed, a group of WT to βc mutant animals was killed 10 wk after transplant, and total protein from BAL fluid was determined. Table 1 shows that the amount of protein in the lungs of WT to βc transplanted animals was indistinguishable from that of unmanipulated WT animals,

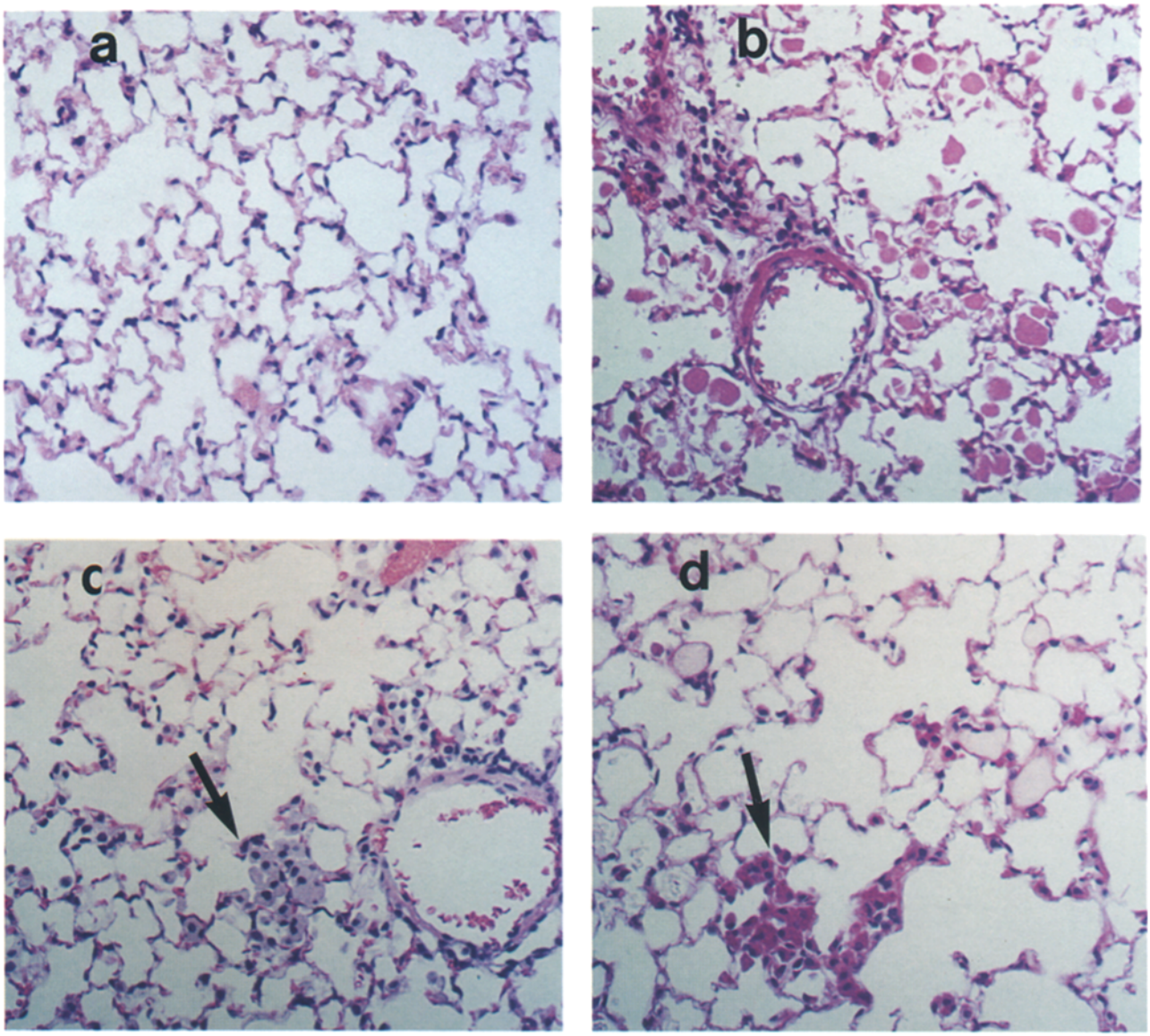

Figure 1. Representative pathology of the lungs of animals evaluated 8 wk after bone marrow transplantation. (*a*) WT to WT transplantation. (*b*) βc mutant to βc mutant transplantation. (*c*) WT to βc mutant transplantation. (*d*) PAS staining of WT to βc mutant transplantation. Arrows in *c* and *d* highlight areas of focal accumulation of macrophages in WT to βc transplantation. Lung sections were stained with hematoxylin and eosin in *a–c*; *d* was stained with PAS.

in contrast to the significantly increased amount of protein in the lungs of βc to βc transplanted animals.

Donor Cell Repopulation of Recipient Lung Tissue. To follow the repopulation of recipient animals with donor-derived cells, we also used WT donor cells that allowed distinction of donor versus recipient cell populations by FACS® analysis (see Materials and Methods). These WT donor cells, marked for identification from recipient cells, reversed PAP in an identical fashion to the above experiments (data not shown). In control stainings, WT F_1 donor (Ly 5.1 × Ly 5.2) and βc mutant (Ly 5.1) peripheral white blood cells showed uniform cell populations for the appropriate Ly 5 alleles (Fig. 3, control panels). After WT to βc mutant animal transplantation, recipient animals contained predominant, but not absolute, donor cell phenotypes in the peripheral blood (Fig. 3, BMT WT→βc KO). The distribution of WT F_1 donor cells in the lung was followed by immunohistochemistry of lung sections using the antibody against Ly5.2. Significant numbers of WT Ly5.2-positive donor cells were detected in the lungs of βc mutant recipients 8 wk after transfer (Fig. 4 *a*), whereas βc to βc (Ly 5.1) transplanted animal lung sections were negative for Ly5.2 staining (Fig. 4 *b*). The morphology and the location of the engrafting Ly 5.2-positive cells suggested alveolar macrophage repopulation, a cell type known to repopulate after bone marrow transplant (8, 9). Consistent with this finding was the reversal of the abnormal macrophage morphology that is characteristic for animals undergoing PAP. Fig. 4 *c* shows alveolar macrophage morphology obtained from the BAL of normal unmanipulated animals, a morphology sim-

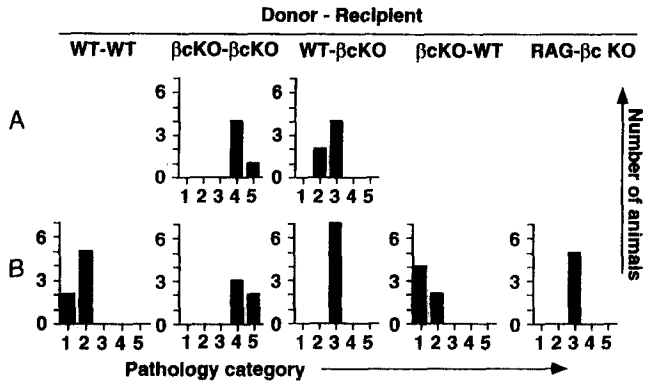

Figure 2. Pathological evaluation of the lungs of bone marrow–transplanted animals. The genotype of bone marrow donor and recipient animals is shown across the top. (*A*) Evaluation 8 wk after transplant. (*B*) Evaluation 12 wk after transplant. In WT-WT and βc KO-WT, two animals from each group were evaluated at 1 yr after transplantation and were included to simplify presentation of the data. All samples were blinded and randomized before evaluation. Histopathological categories were as follows. Category 1: no abnormalities observed. Category 2: mild multifocal accumulation of mononuclear cells around bronchioles and blood vasculature present in some areas; no intraalveolar material present. Category 3: varying degrees of focal accumulations of intraalveolar macrophages and/or type II pneumocytes. Although these cells appear increased in number, due to focal accumulation, their general morphology is normal. Occasional focal areas of interstitial fibrosis or collapsed alveolar septa are present. No acellular intraalveolar material is present. Category 4: ≤90% of the alveolar space is filled with homogeneous PAS-positive and granular precipitate. In some areas, the precipitate is very densely packed. Numerous large vacuolated macrophages contain the granular material and have a foamy appearance. There is a moderate accumulation of perivascular and peribronchiolar mononuclear cells. Category 5: similar to category 4, except that the alveolar space is filled with exclusively densely packed amorphous granular material that appears hyalinized. In addition, there are focal areas of necrosis and type II pneumocyte hyperplasia. The histopathological categories are not meant to indicate a linear scale of pathology, but rather reflect independent and distinct pathological findings.

ilar to alveolar macrophages obtained from βc mutant animals after transplantion with WT bone marrow (Fig. 4 *d*), and in sharp contrast to those obtained from βc mutant animals still undergoing PAP (Fig. 4 *e*).

Given that transfer of WT bone marrow cells into βc mutant mice reversed the course of the lung disease, transfer of βc mutant bone marrow into WT animals may likewise be expected to cause the disease state. However, in four

Table 1. *Protein Accumulation in BAL Fluid*

Group	Total protein in BAL
	mg
WT unmanipulated (n = 6)	≤0.66 ± 0.2
WT-βc mutant (n = 6)	≤0.64 ± 0.083
βc-βc mutant (n = 6)	2.8 ± 0.46

Bone marrow from WT or βc mutant animals was transplanted into βc mutant animals and 10 wk after transplantation total protein content of BAL fluid was determined from each group. Similar results were obtained when testing these samples by the Bradford protein determination method.

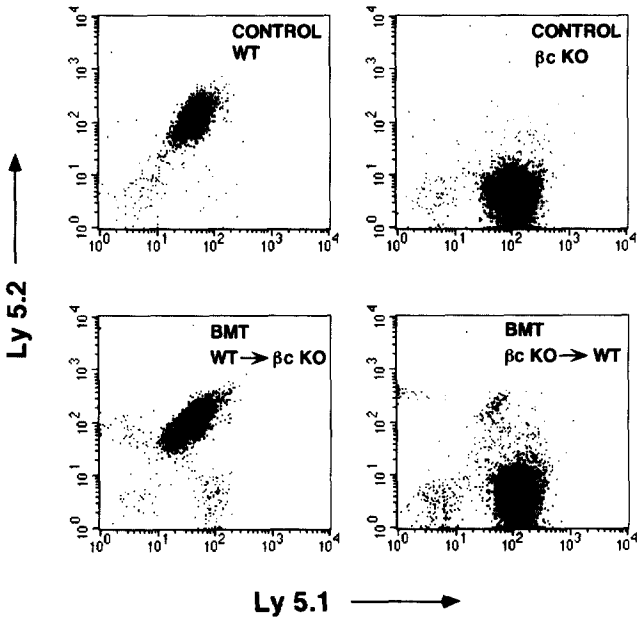

Figure 3. FACS® analysis of peripheral blood cells from control or transplanted animals as described in Fig. 1. WT cells express both Ly 5.1 and Ly 5.2, and βc mutant cells express only Ly 5.1. Each panel describes either control stainings or bone marrow transferred and reconstituted groups.

mice evaluated 12 wk after transfer and in two mice evaluated 1 yr after transfer, we were unable to detect the PAP-like disease (Fig. 2). The blood of these animals was repopulated by donor βc mutant cells, as evidenced by FACS® (Fig. 3, BMT βcKO-WT). The cyto-ablative treatment of the WT recipients by irradiation may not affect cell types such as terminally differentiated macrophages. These remaining radio-resistant cells in the WT animals may be sufficient to prevent the onset of the disease. Alternatively, since bone marrow replacement was not 100% (see Fig. 3), reseeding of the lung with WT precursor cells may be sufficient to prevent the disease.

RAG-2 Mutant Donor Bone Marrow Reversed the PAP Disease. To begin to evaluate which cell type in the bone marrow reversed the disease state in the lung, we used *RAG-2* mutant mice as the source of donor cells. As *RAG-2* mutant animals do not contain lymphocytes (12), the bone marrow from these animals are a source of donor cells more restricted to the myeloid lineage. Indeed, *RAG-2* bone marrow was able to reverse the PAP disease in βc recipient animals 12 wk after transfer (Fig. 2). Animals showed no intraalveolar substance, identical to the findings of the WT (whole bone marrow) to βc mutant animal transplantation.

The data presented in this report document that whole bone marrow from normal animals or bone marrow from *RAG-2* mutant animals can effectively reverse βc-deficient PAP. In previous work we had shown that grossly abnormal alveolar macrophage morphology was a characteristic of mouse βc-deficient PAP (6). This abnormal morphology was corrected after engraftment of donor-derived myeloid lineages into the lungs of βc mutant animals after bone

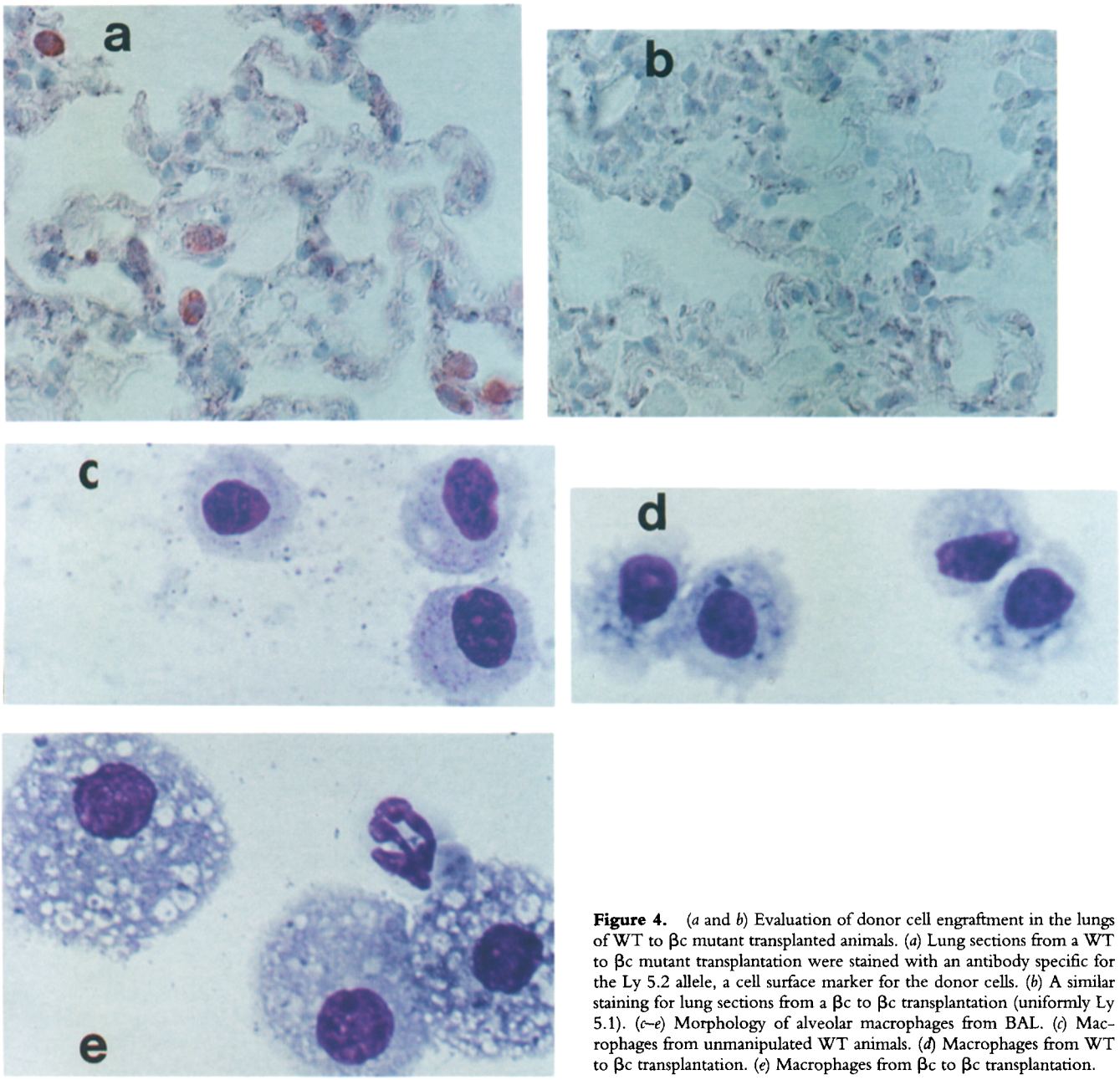

Figure 4. (*a* and *b*) Evaluation of donor cell engraftment in the lungs of WT to βc mutant transplanted animals. (*a*) Lung sections from a WT to βc mutant transplantation were stained with an antibody specific for the Ly 5.2 allele, a cell surface marker for the donor cells. (*b*) A similar staining for lung sections from a βc to βc transplantation (uniformly Ly 5.1). (*c–e*) Morphology of alveolar macrophages from BAL. (*c*) Macrophages from unmanipulated WT animals. (*d*) Macrophages from WT to βc transplantation. (*e*) Macrophages from βc to βc transplantation.

marrow tranplantation, whether or not the donor bone marrow contained the potential to develop lymphocytes. Taken together, these data indicate that a specialized alveolar macrophage dysfunction may cause this disease. A reasonable hypothesis would be a requirement of GM-CSF stimulation of alveolar macrophages to effectively process and/or degrade pulmonary surfactant. Observations of abnormal alveolar macrophages have also been made in the human disease state (13, 14). In support of this hypothesis, Huffman et al. (15) have recently described that transgenic expression of GM-CSF ligand by the lung tissue–specific SP-C promoter corrected PAP when bred to GM-CSF ligand–deficient mice.

In the studies reported here, we used βc mutant recipient animals that were 2–3-mo old at the time of transfer, with the exception of the *RAG-2* transplantation experiment where recipients were 7-mo old. Considering that intraalveolar debris is present at 2 mo and more pronounced at 7 mo in the natural course of the disease, these studies indicate that bone marrow transplantation can not only prevent further progression of the disease, but also reverse a more established disease state.

The ability of bone marrow to effectively reverse mouse PAP caused by loss of βc expression is of significance to the human PAP disease state. A subset of severely affected pediatric PAP patients has been identified with a striking reduction or absence of βc protein expression at the cell surface (Dirksen, U., R. Nishinakamura, P. Groneck, L. Nogee,

R. Murray, and S. Burdach, manuscript submitted for publication). Thus, a subset of humans with this disease, where cells are unresponsive to GM-CSF by a cell-autonomous mutation, may be a candidate for bone marrow transplantation as a means of replacing the defective alveolar macrophage and reversing the disease process. Significantly, engraftment of donor bone marrow into human recipients has been shown by Thomas et al. (10) to result in donor-type alveolar macrophages when evaluated by lung biopsy samples.

The data presented here indicate that βc-deficient mice will serve as a useful model of the PAP disorder, and also emphasize the utility of bone marrow transplantation to correct disease states that may not initially appear to be hematopoietic in nature.

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