# Mycophenolate Mofetil Is Effective and Well Tolerated in the Treatment of Refractory Acute and Chronic Graft-versus-Host Disease

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## Abstract

We enrolled 11 patients with refractory graft-versus-host disease (GVHD) in a prospective trial evaluating the efficacy of mycophenolate mofetil (MMF). Four (67%) of the 6 patients with acute GVHD and all 5 patients with chronic GVHD responded to MMF. Ten (91%) of the 11 patients were able to decrease steroid use (median decrease, 86%; range, 25%-100%). After a median follow-up of 18 months (range, 1-65 months), 7 patients (64%) remained alive. The adverse events were infectious complications (36%), diarrhea (27%), and neutropenia (18%); the only patient discontinuing MMF did so because of grade 4 neutropenia. This preliminary study suggests that MMF is a well-tolerated agent and has a beneficial effect in the treatment of refractory acute and chronic GVHD.
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## 1. Introduction

Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality after allogeneic stem cell transplantation (SCT) [1]. Cyclosporin A (CSA), tacrolimus (FK506), and steroids are effective in the treatment of both acute GVHD and established chronic GVHD [1-3]. However, patients who fail to respond to standard therapy have a poor prognosis [4,5]. The therapeutic options for these patients are limited, and salvage therapies have produced disappointing results to date [6-11].

Mycophenolate mofetil (MMF; CellCept; Roche Diagnostics, Indianapolis, IN, USA) is an ester prodrug of the active immunosuppressant mycophenolic acid, which is a noncompetitive reversible inhibitor of inosine monophosphate dehydrogenase [12-14]. This inhibition blocks the de novo synthesis of guanosine nucleotides, necessary substrates for DNA and RNA synthesis. Lymphocytes depend on this pathway and do not possess the salvage pathways of other cells [14]. This drug has been successfully tested in multicenter randomized trials for preventing renal transplant rejection [15] and has been used in limited trials for the treatment of acute and chronic GVHD [12,16-23]. These retrospective reports have suggested that MMF is an effective agent in these settings. The toxicity profile of MMF, such as upper and lower enteritis, cytopenia, and lack of renal toxicity, is not cross-reactive with the toxicity profiles of CSA, tacrolimus, and steroids, making MMF an attractive candidate for combination therapy.

In February 2000, we began a prospective single-center study in which we analyzed the efficacy of MMF in combination with CSA, tacrolimus, or steroids in the treatment of acute and chronic GVHD in a series of 11 allograft recipients with refractory GVHD.

## 2. Patients and Methods

### 2.1. Patients

Eleven patients with refractory GVHD who had undergone allogeneic SCT between December 1997 and April 2004 were enrolled in this prospective trial. Eligibility criteria were the presence of refractory acute or chronic GVHD after treatment with steroids, CSA, and/or tacrolimus, and the absence of relapse at the time of study enrollment. The protocol received Institutional Review Board approval, and

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signed informed consent was obtained from every patient before study entry.

The patients' characteristics are shown in Table 1. The median age was 46 years (range, 28-66 years). The patients had undergone matched sibling (n = 7), related (n = 3), or unrelated (n = 1) allogeneic transplantation without T-cell depletion. GVHD prophylaxis included CSA and methotrexate for 6 patients, CSA alone for 2 patients, and tacrolimus and methotrexate for 2 patients. Antithymocyte globulin was administered in association with CSA and methotrexate to 1 patient who had received a related transplant mismatched at 2 loci.

## 2.2. GVHD Treatment

The assessment and grading of acute and chronic GVHD were primarily based on clinical findings and were carried out by following the commonly accepted diagnostic criteria [9,10,24,25]. Diagnosis was supported by skin, liver, or gut biopsies whenever indicated and clinically possible. The ocular involvement of chronic GVHD was diagnosed by the Schirmer test. Patient 1 developed skin and liver disease early after cord blood transplantation. The diagnosis of acute GVHD for this patient was based on skin and liver biopsy results, and the patient showed refractoriness to combination treatment with CSA and methylprednisolone (mPSE), suggesting a lower possibility of periengraftment syndrome after cord blood transplantation.

First-line treatment for acute GVHD of grade II or higher or for chronic GVHD consisted of a combination of CSA or tacrolimus with steroids. mPSE was initially administered to patients with acute GVHD of grade II to IV at a dosage of 2 mg/kg per day for 1 to 2 weeks; then the patients were switched to prednisolone (PSE). The tapering schedule for PSE was a dosage reduction of 0.1 to 0.2 mg/kg per week in the responsive cases. PSE was initially administered to patients with chronic GVHD at a dosage of 1 mg/kg and then tapered slowly. If partial or complete resolution of symptoms did not occur or if patients became dependent on steroids (defined as the need for >30 mg/day PSE for more than 6 weeks), they were considered refractory to treatment and were switched to MMF therapy. The blood levels of CSA and tacrolimus of all patients who had been given these drugs reached their target points before MMF treatment was initiated.

MMF was started at a dosage of 1500 mg/day except for 1 patient (no. 1), who received MMF at a dosage of 1000 mg/day because of low body weight (<50 kg) and coexisting pancytopenia. MMF was given orally, and the starting dose was maintained if it was tolerated. Patients were treated with MMF in addition to CSA and steroids (n = 2), tacrolimus and steroids (n = 6), or steroids alone (n = 3). The median time from GVHD onset to the initiation of MMF treatment was 17 days (range, 7-55 days) for acute GVHD and 82 days (range, 59-560 days) for chronic GVHD. The duration of therapy ranged from 30 days to more than 900 days (median, 133 days).

## 2.3. GVHD Monitoring

Response to MMF was assessed for each organ involved, as has been described previously [1,12,18,20]. A complete response was defined as complete resolution of clinical and/or biological signs (skin changes, digestive symptoms, bilirubin level, oral lesions, and joint, lung, and ocular clinical manifestations) that allowed a decrease in dosage or the discontinuation of steroid treatment. A partial response was defined as an improvement in but not a resolution of these clinical and/or biological signs. Stable disease was defined as stable organ involvement. An evaluation of no response referred to the progressive worsening of chronic GVHD. The patients were regularly monitored by full clinical and laboratory evaluations and by pathologic examinations in some cases. Adverse events attributed to MMF were graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0).

## 3. Results

### 3.1. Response to MMF in Refractory Acute GVHD

Response, complication, and survival data from the 6 patients who received MMF treatment for refractory acute GVHD are listed in Table 2. Four (67%) of the 6 patients responded to MMF treatment (Table 2). Although acute GVHD of the gut in patient 3 was resolved by MMF treatment, the patient was considered a nonresponder because of no response to the acute GVHD of the skin and liver. The median time for a patient to show initial signs of response to MMF was 13 days (range, 5-63 days). This interval was calculated as the time to the first objective signs of any improvement, not as the time to maximum response. The responses of these 6 patients according to the involved organs are shown in Figure 1. There was no preference for response according to involved organs.

### 3.2. Response to MMF in Refractory Chronic GVHD

All 5 patients with refractory chronic GVHD responded to MMF therapy and survived thereafter, allowing a decrease in the dosage or discontinuation of steroid treatment in 4 patients (Table 2). The median time to show initial signs of a response was 50 days (range, 27-180 days). Dosage reduction or discontinuation of steroid treatment was possible in 4 of the 5 patients.

### 3.3. Toxicity and Complications

The most common adverse event associated with MMF treatment was diarrhea, which occurred in 3 patients (27%). One patient (no. 9) had to discontinue MMF treatment because of grade 4 neutropenia that was attributed to MMF. Another patient (no. 2) also developed grade 4 neutropenia but required only a dosage adjustment. There were 6 infectious episodes during treatment (cytomegalovirus [CMV] antigenemia, n = 3; CMV pneumonia, n = 1; *Pseudomonas* septicemia, n = 2). The 2 patients with acute GVHD who did not respond to MMF therapy died of progressive acute GVHD and infection. Two other patients experienced relapse of disease while receiving MMF and died of disease progression.

**Table 1.**
Patient Characteristics*

| Patient | Age, y/Sex | Diagnosis | Donor (Sex) | Graft | Conditioning Regimen | GVHD Prophylaxis | Indication to MMF, GVHD Duration before MMF Tx, d | aGVHD Onset Posttransplantation, d/Grade/Sites | aGVHD Tx before MMF (Response) | cGVHD Onset Posttransplantation, d/Grade/Sites | cGVHD Tx before MMF | Concomitant Tx with MMF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 66/M | ALL (CR) | 2 Loci mis, UR (F) | CB | FL/L-PAM/TBI4 | CSA | aGVHD, 12 | 12/IV/skin, liver | CSA, mPSE (NR) | NE | — | CSA, mPSE |
| 2 | 30/F | Marginal zone B-cell lymphoma (PR) | 2 Loci mis, REL (F) | PB | CY/Ara-C/TBI12 | FK506/MTX | aGVHD, 18 | 11/II/skin | FK506, mPSE, PUVA (NR) | No | — | FK506, mPSE |
| 3 | 46/M | CML (CP) | Matched, REL (M) | PB | CY/TBI12 | FK506/MTX | aGVHD, 16 | 21/III/skin, liver, gut | FK506, mPSE (NR) | No | — | FK506, mPSE |
| 4 | 28/M | CML (BC)† | 2 Loci mis, REL (M) | PB | FL/BU | CSA/MTX/ATG | aGVHD, 7 | 8/III/skin, liver | CSA, mPSE (skin, CR; liver, NR) | NE | — | CSA, mPSE |
| 5 | 53/F | Diffuse large B-cell lymphoma (refractory)† | Id sibling (M) | PB | FL/BU | CSA | aGVHD, 44 | 13/III/skin, gut | FK506, mPSE (skin, NR; gut, CR) | No | — | FK506, mPSE |
| 6 | 33/M | Nasal NK/T lymphoma (PR) | Id sibling (F) | PB | CY/TBI12 | CSA/MTX | aGVHD, 55 | 36/III/skin, liver, gut | FK506, PSE (NR) | No | — | FK506, PSE |
| 7 | 61/M | AML (CR) | Id sibling (M) | PB | FL/BU | CSA/MTX | cGVHD, 126 | 25/III/skin, liver, gut | CSA, mPSE (CR) | 82/ext/liver, mouth, ocular | CSA, PSE | PSE |
| 8 | 32/F | AML (CR) | Id sibling (M) | PB | CY/Ara-C/TBI12 | CSA/MTX | cGVHD, 285 | 35/II/skin, liver | CSA, PSE (CR) | 560/ext/skin, liver | PSE | PSE |
| 9 | 32/M | CML (CP) | Id sibling (M) | PB | CY/Ara-C/TBI12 | CSA/MTX | cGVHD, 5 | 25/III/skin, liver, gut | FK506, mPSE (skin/liver, NR‡; gut, CR) | 59/ext/skin | FK506, PSE | FK506, PSE |
| 10 | 52/M | AML from RAEB | Id sibling (F) | PB | CY/Ara-C/TBI12 | CSA/MTX | cGVHD, 1776 | No | — | 79/ext/liver, mouth, ocular | FK506, PSE | FK506, PSE |
| 11 | 59/M | ALL (CR) | Id sibling (M) | PB | FL/BU | CSA/MTX | cGVHD, 235 | No | — | 126/ext/liver | PSE | PSE |

*GVHD indicates graft-versus-host disease; MMF, mycophenolate mofetil; Tx, therapy; aGVHD, acute GVHD; cGVHD, chronic GVHD; ALL, acute lymphoblastic leukemia; CR, complete response; mis, mismatched; UR, unrelated; CB, cord blood; FL, fludarabine; L-PAM, melphalan; TBI, total body irradiation; CSA, cyclosporin A; mPSE, methylprednisolone; NR, no response; NE, nonevaluable; PR, partial response; REL, relative; PB, peripheral blood stem cell; CY, cyclophosphamide; Ara-C, cytarabine; FK506, tacrolimus; MTX, methotrexate; PUVA, psoralen and ultraviolet A irradiation; CML, chronic myeloid leukemia; CP, chronic phase; BC, blast crisis; BU, busulfan; ATG, antithymocyte globulin; Id, identical; NK/T, natural killer/T-cell; PSE, prednisolone; AML, acute myeloid leukemia; ext, extensive; RAEB, refractory anemia with excess of blasts.

†Patient 4 had a history of allogeneic stem cell transplantation, and patient 5 had a history of autologous stem cell transplantation.

‡Progressive type of chronic GVHD of the skin and liver developed subsequently.

**Table 2.**
Response and Toxicity*

| Patient | Response to MMF (Time from MMF Initiation to Response, d) | | | | | | Reduction in Steroid Dosage† | MMF Tx Duration, (Cause of Disruption) | Infections during MMF Tx | Adverse Events (Grade) | Outcome (Cause of Death), Time Posttransplantation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Skin | Liver | Gut | Joints | Ocular | Mouth | | | | | |
| 1 | NR | NR | — | — | — | — | 63% | 30 d (death) | Pseudomonas septicemia, CMV-Ag | No | Dead (aGVHD, Pseudomonas septicemia), 54 d |
| 2 | CR (63) | — | — | — | — | — | 100% | 384 d (efficacy) | CMV-Ag | Neutropenia (4)‡ | Alive CR, 26+ mo |
| 3 | NR | NR | CR (7) | — | — | — | 80% | 111 d (death) | Pseudomonas septicemia, CMV pneumonia | No | Dead (aGVHD, CMV pneumonia), 147 d |
| 4 | — | CR (5) | — | — | — | — | 87% | 204 d (relapse) | CMV-Ag | No | Dead (relapse), 209 d |
| 5 | CR (10) | — | — | — | — | — | 90% | 89 d (relapse) | No | No | Dead (relapse), 145 d |
| 6 | CR (15) | CR (15) | CR (15) | — | — | — | 80% | 133 d (efficacy) | No | diarrhea (3)‡ | Alive CR, 68+ mo |
| 7 | — | CR (70) | — | — | CR (70) | CR (360) | 100% | 825 d (efficacy) | No | No | Alive CR, 25+ mo |
| 8 | PR (57) | SD | — | — | — | — | 25% | 110 d (efficacy) | No | Diarrhea (1)‡ | Alive CR, 66+ mo |
| 9 | SD | PR (27) | — | — | — | — | 85% | 56 d (neutropenia) | No | Neutropenia (4)‡ | Alive CR, 64+ mo |
| 10 | — | PR (180) | — | — | PR (180) | PR (180) | 0% | 30+ mo | No | Diarrhea (1)‡ | Alive CR, 91+ mo |
| 11 | — | CR (31) | — | — | — | — | 100% | 16+ mo | No | No | Alive CR, 27+ mo |

*CMV-Ag indicates cytomegalovirus antigenemia; SD, stable disease. Other abbreviations are expanded in the first footnote to Table 1.
†Percent reduction in steroid dosage at the end of MMF treatment or at last follow-up.
‡Neutropenia in patients 2 and 9 was resolved with a reduction in MMF dosage and MMF discontinuation, respectively. Diarrhea in patients 6, 8, and 10 was resolved with supportive medication.

## 4. Discussion

Even with the best immunosuppressive regimens using CSA, tacrolimus, and steroids, many patients still succumb to acute and chronic GVHD. These patients are likely to die of GVHD itself or from infectious complications secondary to prolonged immunosuppression, as well as to the depression of their immune system by GVHD [1-11]. We attempted to improve the prognosis of such patients by combining MMF with other commonly used immunosuppressive agents. Four (67%) of 6 patients with refractory acute GVHD responded with no subsequent development of chronic GVHD, and MMF therapy was eventually stopped in 2 of these patients because of successful outcomes. Additionally, all 5 patients with refractory chronic GVHD who were treated with MMF showed improvements of clinical symptoms, and MMF was discontinued in 2 patients. These results seemed comparable to the outcomes reported for previous studies on treatment of acute GVHD (response rates, 31%-71%) and chronic GVHD (response rates, 46%-77%) [12,16-23]. In addition, the administration of MMF allowed a dosage reduction of steroids in 10 of the 11 patients. The remaining patient (no. 10), who had been treated with a combination of 7.5 mg PSE daily and the maximum dose of tacrolimus before the initiation of MMF therapy, became free of tacrolimus treatment despite continuing the same PSE dosage thereafter. These findings suggest that MMF may be an effective salvage treatment for refractory GVHD.

Although all 5 patients with chronic GVHD in the current study have maintained good clinical conditions after the initiation of MMF treatment, only 2 patients (33%) with acute GVHD have survived. The difference between the 2 groups in the rate of response to MMF may partly account for this observation. Another explanation is that 4 of the 6 patients with acute GVHD had advanced disease at the time of transplantation, whereas only 1 of the 5 patients with chronic GVHD had advanced disease.

Several reports have shown that the response to MMF developed within 2 months after MMF introduction, irrespective of whether acute or chronic GVHD was targeted [20,22,23]. The median time for a patient to show initial signs of response to MMF treatment was 31 days (range, 5-180 days) in the present study. This interval was the time to the start of any improvement and not the time to maximum response. Of note is that 3 (33%) of 9 responders began to show improvements in GVHD more than 2 months after MMF initiation (at 63, 70, and 180 days). These findings suggest that MMF should be continued for at least 3 months to provide an opportunity for late responses to develop.

MMF was generally well tolerated. Of note is that treatment with MMF was not discontinued for adverse events except in a single patient who responded to MMF but experienced grade 4 neutropenia that required the discontinuation of MMF therapy. Other adverse events were resolved with supportive medication or by reducing the MMF dosage. Our findings may serve to strengthen the advantage of MMF, which causes a relatively small number of adverse events including nephrotoxicity and liver toxicity compared with other new immunosuppressive drugs [19].
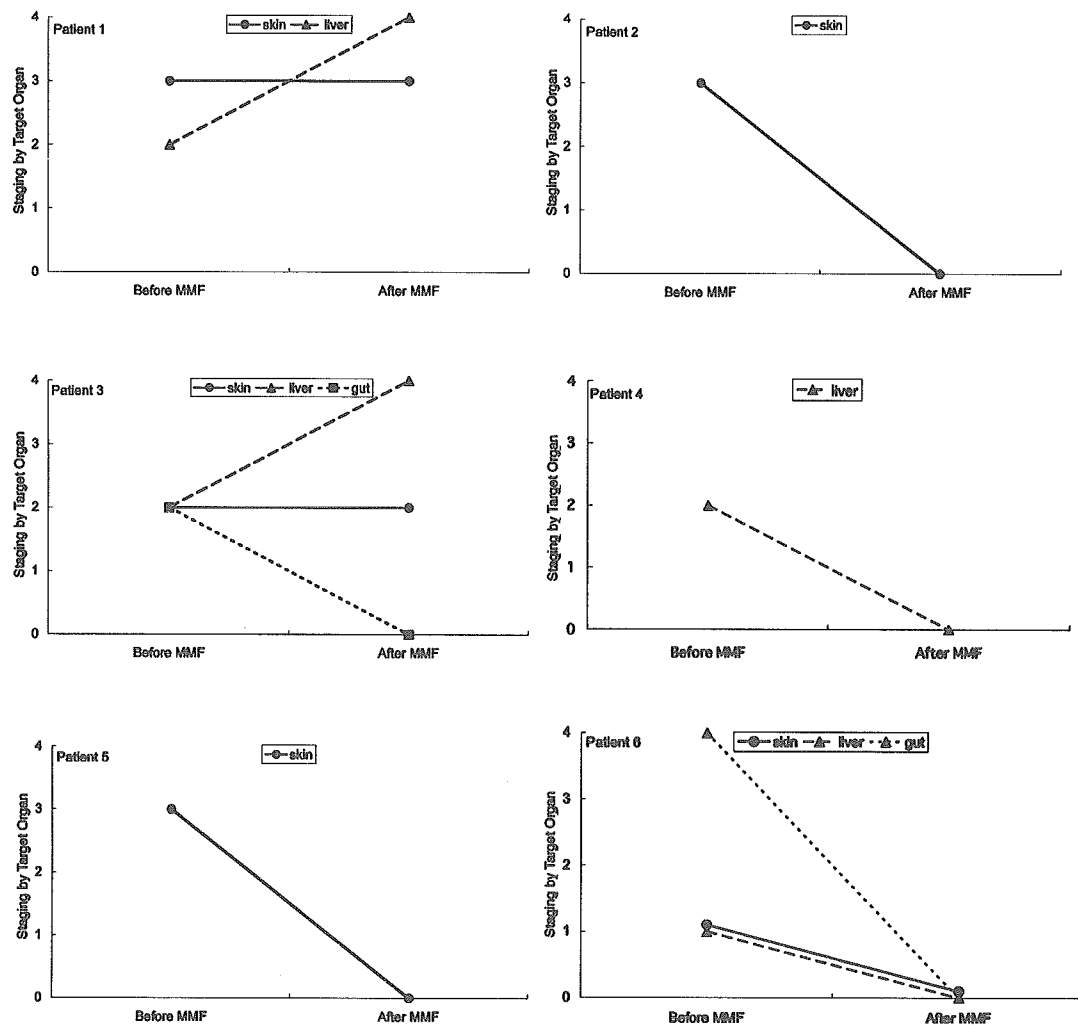
**Figure 1.** Response to mycophenolate mofetil (MMF) by target organ in 6 patients with acute graft-versus-host disease.

Six opportunistic viral or bacterial infections occurred in 4 of the patients. Two patients died from infection (*Pseudomonas* septicemia in one patient and CMV pneumonia in the other) coinciding with progressive acute GVHD, which developed while these patients received MMF. These findings may be consistent with previous reports that the use of MMF in allogeneic SCT was an independent risk factor for CMV infection [26] and was associated with a high risk of serious viral or bacterial infections [20,22]. However, it is difficult to accurately ascertain the negative impact of MMF on infectious complications in such a small retrospective study that lacks a comparison group in terms of salvage GVHD treatment.

In the current study, 2 patients relapsed during MMF therapy, although both patients were at high risk of relapse at the time of transplantation (Tables 1 and 2). Recently, Shapira et al [27] reported that MMF does not impair graft-versus-leukemia (GVL) effects or reduce lymphokine-activated killer cell activity in mice, whereas CSA had already been shown in mice [28] and in clinical practice [29] to suppress

the GVL effects inducible by allogeneic donor lymphocytes. A study that compared tacrolimus with CSA for GVHD prophylaxis has shown that the relapse rate among recipients of HLA-matched transplants from siblings was significantly higher in the tacrolimus group than in the CSA group [30], indicating that tacrolimus may compromise the GVL effects more significantly than CSA. However, whether MMF treatment is irrelevant to disease relapse is still unknown.

No patients in the current study developed thrombotic microangiopathy (TMA) during treatment with MMF. TMA is a syndrome of microangiopathic hemolytic anemia, thrombocytopenia, and renal dysfunction [31]. The association of TMA with immunosuppressive agents given after SCT, such as CSA, tacrolimus, and sirolimus, is well established [31,32]. Despite the unknown etiology of TMA, the pathologic finding of endothelial injury is commonly seen in patients with TMA. Of note is that no literature review has reported that MMF induces endothelial toxicity. These findings suggest that MMF, if used instead of CSA and tacrolimus, could have a benefit in decreasing the risk of TMA after SCT.

These preliminary results support the hypothesis that MMF can be used safely and has encouraging efficacy in the treatment of patients with GVHD who fail to benefit from conventional therapy. We emphasize that our results may have been influenced by the small number of patients in this study, and it is difficult to draw a final conclusion. In addition, MMF reduced the requirement for steroids, thereby reducing the risk of complications due to iatrogenic immunosuppression. A prospective randomized clinical trial is warranted to assess the impact of MMF in the treatment of refractory GVHD. The early combination of MMF with other treatment strategies may further improve the response rate and survival of these patients. Additional studies are also needed to test this hypothesis.

## Acknowledgments

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### Stem Cell Transplantation

## High incidence of cytomegalovirus reactivation in adult recipients of an unrelated cord blood transplant

**This retrospective analysis for cytomegalovirus (CMV)-seropositive adult transplant recipients showed that CMV antigenemia occurred after transplantation in 10/10 (100%) recipients of unrelated cord blood, 17/39 (43%) recipients of a related matched donor graft, 16/23 (79%) recipients of an unrelated matched donor graft, and 8/12 (67%) recipients of a mismatched related donor graft. These results suggest that unrelated cord blood transplantation itself may be correlated with a high incidence of CMV reactivation.**

Cytomegalovirus (CMV) infection is still a major concern following allogeneic hematopoietic transplantation because CMV pneumonia is fatal in 70% of patients, even when treated with a combination of antiviral therapies and CMV hyperimmune immunoglobulin.[1] Allogeneic cord blood transplantation, especially from unrelated donors, has progressively gained favor as treatment for patients with both malignant and non-malignant disorders.[2-4] As compared to allogeneic bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT), advantages of unrelated cord blood transplantation (UCBT) include ease and safety of cell collection, low risk of transmitting viral infections, prompt availability of stem cells, and reduced incidence and severity of graft-versus-host disease (GVHD).[2-4] The reduction of GVHD after UCBT is likely due to the naïve state of cord blood lymphocytes and the low cytotoxic capacity of cord blood T cells.[5] However, such immunological immaturity after UCBT can place a patient at risk

Table 1. Patient characteristic.

| Characteristics | Stem cell donor | | | |
| --- | --- | --- | --- | --- |
| | HLA identical sibling | HLA matched unrelated donor | HLA mismatched relative | Unrelated CB |
| No. of patients | 39 | 23 | 12 | 10 |
| Sex, male/female | 23/16 | 10/13 | 5/7 | 6/4 |
| Median age (range), years | 53 (14-69) | 36 (17-54) | 43 (15-58) | 61 (15-69) |
| Disease | | | | |
| Acute myelogenous leukemia | 9 | 6 | 0 | 2 |
| Acute lymphoblastic leukemia | 4 | 6 | 6 | 2 |
| Chronic myeloid leukemia | 3 | 4 | 2 | 0 |
| Myelodysplastic syndrome | 6 | 2 | 0 | 1 |
| Non-Hodgkin's lymphoma | 7 | 3 | 3 | 1 |
| Sever aplastic anemia | 4 | 2 | 1 | 1 |
| Myelofibrosis | 1 | 0 | 0 | 0 |
| Renal cell carcinoma | 4 | 0 | 0 | 3 |
| Osteosarcoma | 1 | 0 | 0 | 0 |
| Standard risk/advanced risk* | 18/21 | 14/9 | 3/9 | 1/9 |
| Stem cell source PBSC/BM | 33/6 | 0/23 | 10/2 | 0/0 |
| HLA disparity 0/1/2/3 | 39/0/0/0 | 23/0/0/0 | 0/2/6/3/1 | 3/1/6/0 |
| CMV-seropositive donor | 35 | 22 | 11 | 0 |
| Prior transplantation | 4 | 2 | 1 | 5 |
| Conditioning regimen Myeloablative/Reduced-intensity | 15/24 | 18/5 | 7/5 | 1/9 |
| GVHD prophylaxis CSP-based/FK506-based | 38/1 | 11/11 | 7/5 | 7/3 |
| Use of ATG | 5 | 3 | 2 | 1 |
| Use of steroids | 13 | 4 | 8 | 6 |
| Use of MMF | 3 | 0 | 5 | 5 |
| Survival >100 days, % | 92 | 87 | 75 | 78 |
| Survival >365 days, % | 82 | 83 | 40 | 56 |

PBSC, peripheral blood stem cell; BM, bone marrow; CB, cord blood; CSP, cyclosporine; FK506, tacrolimus; MMF, mycophenolate mofetil. *Acute leukemia in first remission, chronic myeloid leukemia in the first chronic phase, myelodysplastic syndrome with refractory anemia or refractory anemia with ringed sideroblasts, malignant lymphoma in any remission, and aplastic anemia were defined as standard-risk diseases. All other patients were classified as having advanced disease.

of early infectious complications, accounting for most transplant-related deaths, especially in adults.[1,6] We have observed that patients undergoing UCBT appear to be at increased risk of CMV infection. Ninety-one consecutive adult patients who were CMV-seropositive and received non-T-cell-depleted allogeneic transplants at the Kanazawa University Hospital between April 1999 and April 2004 were eligible for inclusion. Written informed consent was obtained from all patients. Six patients died of regimen-related toxicities before engraftment and one developed primary graft rejection followed by autologous hematopoietic recovery. The remaining 84 patients had

**Table 2. Acute GVHD and CMV infection according to stem cell donor.**

| | Stem cell donor | | | |
| --- | --- | --- | --- | --- |
| | HLA-identical sibling | HLA-matched unrelated donor | HLA-mismatched relative donor | Unrelated CB |
| II-IV acute GVHD | 14/39 (36) | 8/23 (35) | 8/12 (67) | 5/10 (50) |
| CMV antigenemia (%) | 17/39 (44) | 16/23 (70) | 8/12 (67) | 10/10 (100) |
| Days between transplantation and first antigenemia, median (range) | 43 (20-99) | 29 (18-47) | 38.5 (5-95) | 32.5 (0-42) |
| Days between final and first antigenemia, median (range) | 14 (1-117) | 21.5 (0-80) | 94 (0-161) | 60 (7-104) |
| Peak no. of CMV-positive cells among 5x10⁴ leukocytes, median (range) | 10 (1-395) | 8 (1-714) | 15 (4-250) | 46 (7-543) |
| CMV disease (%) | 1/39 (3) | 1/23 (4) | 0/12 (0) | 1/10 (10) |
| Late CMV antigenemia (%) | 3/36 (9) | 1/19 (5) | 5/9 (56) | 3/7 (43) |

successful initial engraftment and were included in the analysis. The patients' characteristics are given in Table 1. CMV antigenemia assays were carried out as previously described.[7,8] In brief, heparinized blood samples were fractionated by dextran sedimentation. Slides were prepared in duplicate after cytocentrifugation; $1.5 \times 10^5$ leukocytes were fixed with formaldehyde and stained with HRP-C7 monoclonal antibodies that specifically bind the pp65 antigen of CMV (Teijin, Tokyo, Japan). The degree of CMV antigenemia was expressed as the number of CMV antigen-positive cells per $5 \times 10^4$ leukocytes. For the evaluation of CMV antigenemia, $5 \times 10^4$ leukocytes were always analyzed, because the detection limit was one CMV antigen-positive cell per $5 \times 10^4$ leukocytes in this assay.[7,8] CMV antigenemia was defined as $\geq 1$ antigen-positive cell.[7,8] For the diagnosis of CMV disease, such as pneumonia, gastroenteritis, retinitis, and hepatitis, the CMV antigenemia had to be accompanied by clinical symptoms, signs, and histologic confirmation.[9] Late CMV antigenemia was defined as that occurring after day 100. Ganciclovir or foscarnet was used as pre-emptive therapy to prevent CMV disease. The decision to use pre-emptive therapy was based entirely on a positive antigenemia test ($\geq 3$ antigen-positive cells/$5 \times 10^4$ leukocytes).[7,8] Ganciclovir was administered as an intravenous infusion at the dose of 5 mg/kg/b.i.d. Neutropenic patients (absolute neutrophil count, less than 750/µL) were given foscarnet instead of ganciclovir; the induction dose of foscarnet was 60 mg/kg intravenously every 12 hours, followed by maintenance doses of 90 mg/kg once daily.[10] Treatment was stopped if two consecutive CMV

antigenemia assays were negative. Granulocyte colony-stimulating factor was administered when the absolute neutrophil count was <500/µL. Previous reports demonstrated the high sensitivity of the HRP-C7 assay and validated the analyzed cell count and the cut-off we relied on in our study.[7,8]

All UCBT recipients developed CMV antigenemia whereas 44% of the recipients of related matched donor grafts, 70% of the recipients of unrelated matched donor grafts, and 67% of those receiving mismatched related donor transplants did so (Table 2). CMV-associated disease occurred in three patients (4%), gastroenteritis in two and interstitial pneumonia in one. Of these three patients only one patient, who developed interstitial pneumonia after UCBT, died of CMV disease. Forty-one patients (80%) received antiviral therapy; ganciclovir was used in 20 patients, foscarnet in 5, and the combination of both in 16. In the remaining 10 patients, CMV antigenemia remained below the detection level and disappeared without antiviral therapy.

Although our data still require confirmation in a larger prospective study, the impact of UCBT on the development of CMV antigenemia might be considered when designing future transplant strategies, at least until more effective methods for prophylaxis of CMV reactivation become available.

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*Stem Cell Transplantation*

## Two allogeneic hematopoietic stem cell transplantations without the use of blood-product support

We successfully performed two allogeneic hematopoietic stem cell transplantations from matched unrelated donors without the use of blood-product support after treosulfan-based conditioning in two women with acute myeloid leukemia who were Jehovah's witnesses and refused transfusions of blood products.

In the last two years we were confronted with a mother and her daughter with acute myelogenous leukemia (AML) who were both members of the community of Jehovah's witnesses, a religious group that refuses transfusion of any major blood product.

Despite their religious objection to blood products we offered both induction chemotherapy and allogeneic hematopoietic stem cell transplantation as consolidation therapy, which they accepted. We felt able to propose this strategy for two reasons: (i) based on our experience with a stringent therapeutic platelet transfusion protocol that we have developed during the last years, we know that severe thrombocytopenia can be managed without prophylactic platelet transfusion. In more than 200 patients (during induction chemotherapy for AML or after autologous peripheral stem cell transplantation) we have shown that a therapeutic transfusion strategy is safe. In one third of our patients autologous transplantation could be performed without any platelet transfusions. Bleeding complications among patients transfused on demand were completely comparable to those among our former patients who received prohylactic platelet transfusions at a trigger platelet count of $10 \times 10^9/L$;[1,2] (ii) we used allogeneic stem cell transplantation after a reduced toxicity conditioning regimen as consolidation treatment since hematologic regeneration could be expected to be significantly quicker than after repeated cycles of high-dose cytosine arabinoside as consolidation. The same is true for autologous transplantation because stem cells should be collected only after a minimum of two intensive courses of chemotherapy as *in vivo*

**Table 1. Patients' characteristics and follow-up.**

| | Mother | Daughter |
|---|---|---|
| Diagnosis | AML-M1 | AML-M2 |
| Karyotype | Normal female | Normal female |
| Status of remission before transplantation | CR | CR |
| Age at transplantation | 48 | 21 |
| Conditioning regimen | TBI/Fludarabine/Treosulfan | TBI/Fludarabine/Treosulfan |
| Donor | Matched unrelated male | Matched unrelated male |
| Blood group P/D | A Rh+/A Rh+ | A Rh+/A Rh+ |
| CMV | P⁻/D⁻ | P⁻/D⁻ |
| Source of stem cells | Bone marrow | Peripheral blood |
| No. of transplanted cells (CD34×10⁶/kg) | 1.64 | 8.4 |
| Immunosuppression (oral) | CSA/MMF(4×500mg) | CSA/MMF(4×500mg) |
| Hematological toxicity | | |
| Leukocytes <1.0×10⁹/L (days) | 6 | 14 |
| Neutrophils <0.5×10⁹/L (days) | 12 | 18 |
| Platelets <20×10⁹/L[<10×10⁹/L] | 2 [0] | 3 [0] |
| Minimal hemoglobin (g/dL) | 11 | 9 |
| Chimerism analysis (FISH) | > 90% donor, day +15, ongoing | > 90% donor, day +12, ongoing |

TBI: total body irradiation; P: patient; D: donor; CSA: cyclosporine A; MMF: mycophenolatmofetil; FISH: fluorescent in situ hybridization.

purging. The risks of graft-versus-host disease (GVHD) after allogeneic transplantation and its higher probability of cure had to be weighed against the greater hematologic and non-hematologic toxicity of the alternative procedures.

In the daughter we favored allogeneic transplantation despite normal cytogenetics because her AML was diagnosed as a first relapse after a chemotherapy-treated AML as a child more than 10 years previously. The mother was informed that allogeneic transplantation from a matched unrelated donor is not standard therapy in AML in first remission without high-risk cytogenetics. Both patients were informed on the extraordinary risks of refusing blood transfusions during the treatment of AML. Both patients gave their written informed consent.

The characteristics of the patients, their treatment and the follow-up are shown in Table 1. A complete remission was achieved after dose-reduced induction chemotherapy with daunorubicin (50 mg/m²×2) and cytosine arabinoside (100 mg/m² for 5 days as a continuous infusion). Once HLA-identical unrelated donors had been identified for each patient we started conditioning in both with a combination of a marrow ablative dose of treosulfan (3×10 g/m²)

# Minor population of CD55−CD59− blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia

Chiharu Sugimori, Tatsuya Chuhjo, Xingmin Feng, Hirohito Yamazaki, Akiyoshi Takami, Masanao Teramura, Hideaki Mizoguchi, Mitsuhiro Omine, and Shinji Nakao

We investigated the clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria (PNH)–type blood cells in patients with acquired aplastic anemia (AA). We quantified CD55−CD59− granulocytes and red blood cells (RBCs) in peripheral blood from 122 patients with recently diagnosed AA and correlated numbers of PNH-type cells and responses to immunosuppressive therapy (IST). Flow cytometry detected 0.005% to 23.1% of GPI-AP− cells in 68% of patients with AA. Sixty-eight of 83 (91%) patients with an increased proportion of PNH-type cells (PNH+) responded to antithymocyte globulin (ATG) + cyclosporin (CsA) therapy, whereas 18 of 39 (48%) without such an increase (PNH−) responded. Failure-free survival rates were significantly higher (64%) among patients with PNH+ than patients with PNH− (12%) at 5 years, although overall survival rates were comparable between the groups. Numbers of PNH-type and normal-type cells increased in parallel among most patients with PNH+ who responded to IST, suggesting that these cells are equally sensitive to immune attack. These results indicate that a minor population of PNH-type cells represents a reliable marker of a positive IST response and a favorable prognosis among patients with AA. Furthermore, immune attack against hematopoietic stem cells that allows PNH clonal expansion might occur only at the onset of AA. (Blood. 2006;107:1308-1314)

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## Introduction

Immunosuppressive therapy (IST) with antithymocyte globulin (ATG) plus cyclosporin (CsA) is the standard approach to treating acquired aplastic anemia (AA).[1-5] Approximately 70% of patients respond to this therapy and achieve remission. However, for the remaining 30%, IST might even be harmful because of an increased risk of opportunistic infections, particularly in the absence of any remission. The immune pathophysiology of patients should thus be understood at diagnosis, and IST should be applied only to those with immune-mediated AA. Several factors have been proposed as good markers that appear to reflect the immune pathophysiology of AA. These factors include an increased ratio of activated T cells,[6] increased interferon-γ expression in bone marrow,[7] and peripheral-blood T cells,[8] as well as increased expression of heat-shock protein 70.[9] Although these markers are useful in predicting responses to IST, few patients with AA have been tested, and the assays applied to detect these abnormalities are vulnerable to the effects of artifacts and the transportation of test samples. Consequently, none of the markers have been practically applied to predict responses to IST. Because of this, patients with AA are placed on IST without understanding the underlying pathophysiology.

One marker closely associated with immune pathophysiology in bone marrow failure is a small number of cells that are glycosylphosphatidylinositol-anchored membrane protein-deficient (GPI-AP−), namely paroxysmal nocturnal hemoglobinuria (PNH)–type cells.[10-14] Dunn et al[11] have demonstrated that an increase in CD15− CD66b−CD16+ granulocytes is associated with a good response to ATG among patients with myelodysplastic syndrome (MDS). Using 2-color flow cytometry that can distinguish proportions of CD55−CD59−CD11b+ granulocytes and CD55−CD59− glycophorin A+ red blood cells (RBCs) below 0.1%, we also demonstrated that a population of 0.01% to 6% PNH-type cells among granulocytes and red blood cells predicts a response to CsA in patients with MDS.[15] Although one study group did not find a correlation between PNH-type cells and response to ATG in patients with AA,[14] an increase in the proportion of PNH-type cells was correlated with a good response to IST among our patients with AA[16] as well as those in another report.[12] However, the significance of a minor population of PNH-type cells in the management of patients with AA has remained obscure because the number of patients with recently diagnosed AA has been small and follow-up periods have not been long enough. Our sensitive flow cytometric protocol has not become popular despite its potential clinical usefulness, perhaps because of the lower cut-off values (0.003% for granulocytes and 0.005% for RBCs) than previous assays.[11,12,17,18]

The outcome of IST in patients with AA is negatively affected by the length of time from diagnosis to treatment.[19] To clarify the role of a marker that would predict a good response to IST, the marker should be tested on patients who have been recently diagnosed with AA and before they receive therapy, and then the marker should be correlated with the subsequent response to IST. Since 1999, we have been studying the presence of PNH-type cells in peripheral blood using flow cytometry in 241 patients who had not yet undergone therapy and who were diagnosed with AA. The present study focuses on 122 patients who were treated with ATG and CsA within 1 year of the diagnosis of AA and compares the response rates to IST and subsequent survival between patients with (PNH⁺) and without (PNH⁻) an increased proportion of PNH-type cells. We also examined changes in the number of PNH-type cells after successful IST to characterize the immune system attack against hematopoietic stem cells that confers a survival advantage on PNH-type stem cells in immune-mediated AA.

## Patients, materials, and methods

### Patients

We evaluated PNH-type cells in peripheral-blood samples from 122 Japanese patients (55 men and 67 women; median age, 56 years) with idiopathic AA (75 severe and 47 moderate AA) before they received IST. The patients were diagnosed with AA at Kanazawa University Hospital, hospitals participating in a cooperative study led by the Intractable Disease Study Group of Japan, and other referring institutions. The severity of AA was classified according to the criteria proposed by Camitta et al.[20] All patients were treated with ATG Lymphoglobuline (Aventis Behring, King of Prussia, PA) 15 mg/kg/d, 5 days; plus CsA (Novartis, Basel, Switzerland) 6 mg/kg/d; within 1 year of diagnosis between April 1999 and December 2004. The dose of CsA was adjusted to maintain trough levels between 150 and 250 ng/mL, and the appropriate dose was administered for at least 6 months. Granulocyte colony-stimulating factor (G-CSF; filgrastim, 300 μg/m² or lenograstim, 5 μg/kg) was administered to some patients. Response to IST was evaluated according to the response criteria described by Camitta.[21] Complete response (CR) was defined as hemoglobin normal for age, neutrophil count more than $1.5 \times 10^9$/L, and platelet count more than $150 \times 10^9$/L. Partial response (PR) was defined as transfusion independent and no longer meeting criteria for severe disease in patients with severe AA, and it was defined as transfusion independence (if previously dependent) or doubling or normalization of at least one cell line or increase in baseline hemoglobin of more than 30 g/L (if initially less than 60 g/L), neutrophil count of more than $0.5 \times 10^9$/L (if initially less than $0.5 \times 10^9$/L), and platelet count of more than $10 \times 10^9$/L (if initially less than $20 \times 10^9$/L) in patients with moderate AA. The patients provided written, informed consent to participate in all procedures associated with the study, which was reviewed and approved by the ethical committee of Kanazawa University Hospital (study no. 46). The study also conforms to the recently revised tenets of the Helsinki protocol.

### High-resolution 2-color flow cytometry

We improved the 2-color flow cytometry developed by Araten et al[22] as follows. Briefly, 3 to 5 mL heparinized blood was drawn from each patient. To detect PNH-type granulocytes, RBCs were lysed in $NH_4Cl$ 8.26 g/L, $KHCO_3$ 1.0 g/L, and EDTA · E4Na 0.037 g/L (lysis buffer). After a saline wash, 50 μL leukocyte suspension was incubated with 4 μL phycoerythrin (PE)–labeled anti-CD11b monoclonal antibodies (mAbs; Becton Dickinson, Franklin Lakes, NJ), fluorescein-isothiocyanate (FITC)–labeled anti-CD55 mAbs (clone IA10, mouse IgG2a; Pharmingen, San Diego, CA), and FITC-labeled anti-CD59 mAbs (clone p282, mouse IgG2a; Pharmingen) on ice for 30 minutes.[13] To detect PNH-type RBCs, PE-labeled anti-glycophorin A mAbs (clone JC159; DAKO, Glostrup, Denmark) were

included instead of anti-CD11b mAbs.[15] Fresh blood was diluted to 3% in phosphate-buffered saline (PBS), and then 50 μL was incubated with 4 μL PE-labeled anti–glycophorin A mAbs, FITC-labeled anti-CD55, and anti-CD59 mAbs on ice for 30 minutes. A total of at least $1 \times 10^5$ CD11b⁺ granulocytes and glycophorin A⁺ RBCs within each corresponding gate were analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ) flow cytometry. To exclude damaged cells that often produce false-positive results, all samples were treated for flow cytometry within 24 hours after collection, and SSC^dim and CD11b^dim granulocytes and glycophorin A^dim RBCs on the histograms were excluded from the analyses by careful gating as shown in Figure 1A. On the basis of analytic results from 68 healthy individuals, the presence of greater than 0.003% CD11b⁺ granulocytes and 0.005% glycophorin A⁺ RBCs was considered abnormal. Both thresholds greatly exceeded the mean + 4 SDs for GPI-AP⁻ granulocytes (0.0025%) and RBCs (0.0032%) determined in healthy individuals.[13,15] When PNH-type cells were increased in only 1 of the 2 cell lineages, another sample was collected, and the patient was deemed PNH⁺ only when the second sample produced similar results.

We compared the sensitivity of detecting a few PNH-type cells in this manner with that of a low-resolution method[23] by analyzing the blood of some patients by 2-color flow cytometry using both PE-labeled anti-CD55
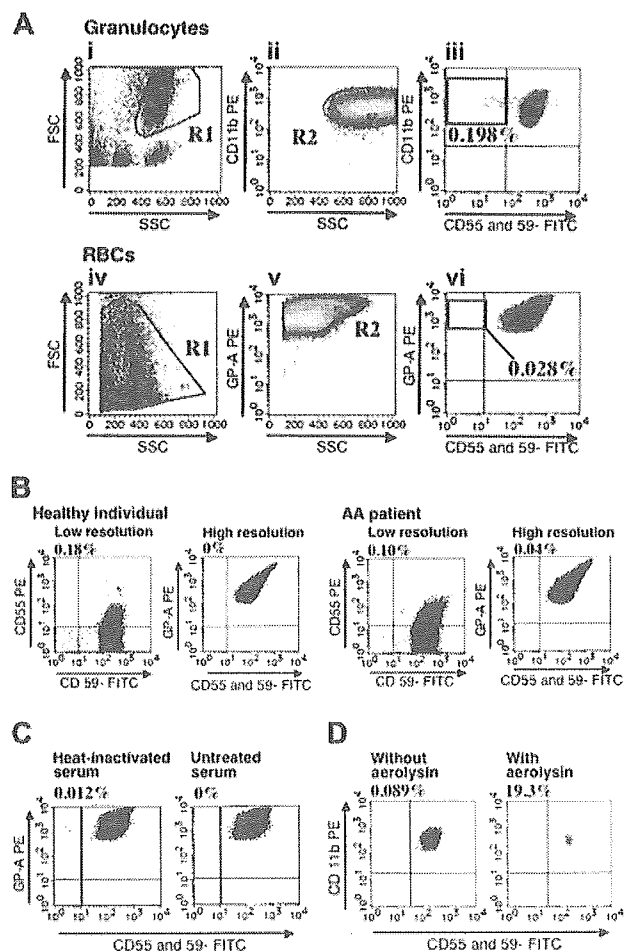


**Figure 1. Validity of high-resolution flow cytometry.** (A) An example of analysis on a patient with PNH⁺ AA is shown. Gates were set up to exclude SSC^dim (i) and CD11b^dim granulocytes and glycophorin A^dim RBCs (ii,v). Cells within rectangles showing horizontal distribution represent PNH-type cells. (B) RBCs from a healthy individual and a patient with AA were examined using a low-resolution assay and the high-resolution assay. Numbers on histograms denote the percentages of CD55⁻CD59⁻ cells in total RBCs for the low-resolution assay, and in glycophorin A⁺ RBCs for the high-resolution assay. (C) RBCs from a patient with PNH⁺ AA were incubated in acidified saline containing heat-inactivated or untreated serum. CD55⁻CD59⁻ RBCs were then quantified. (D) PNH⁺ AA WBCs were incubated with or without $0.5 \times 10^{-8}$ M aerolysin and analyzed by flow cytometry.

and FITC-labeled anti-CD59 mAbs. This assay defines the presence of 1% or more PNH-type cells as a significant increase.

## Modified Ham test

Peripheral blood of patients with AA with a low proportion (< 0.1%) of CD55⁻CD59⁻ RBCs was washed with saline and suspended in saline at a hematocrit of 50%. The RBC suspension (15 μL) was incubated with 80 μL heat-inactivated fetal calf serum (FCS) for 10 minutes at 4°C for sensitization by anti–human heteroantibodies and then washed with saline. Human AB serum as a source of complement (0.5 mL) and 55 μL 0.2 N HCl were then added to the cell suspension. The negative control included heat-inactivated human AB serum instead of untreated human AB serum. These RBC suspensions were incubated for 60 minutes at 37°C and washed with PBS, and then the RBCs were analyzed by flow cytometry as described in "High resolution 2-color flow cytometry."

## Aerolysin treatment of granulocytes

Peripheral blood from patients with AA with a low proportion of PNH-type granulocytes was lysed as described in "High resolution 2-color flow cytometry," and suspended in PBS at a density of 2 × 10⁵ cells/mL. The leukocyte suspension was split into 2 portions; one was incubated for 15 minutes with and the other without 0.5 × 10⁻⁸ M aerolysin at 37°C.[24] Before and after the incubation with aerolysin, the suspension was examined by flow cytometry to detect CD55⁻CD59⁻CD11b⁺ granulocytes as described in "High resolution 2-color flow cytometry."

## Statistics

The Mann-Whitney test compared clinical characteristics between patients with PNH⁺ and patients with PNH⁻. Fisher exact test and logistic regression modelling[25] analyzed associations between individual pretreatment variables with response to IST. Kaplan-Meier methods graphically compared the cumulative incidence of the response with IST and time to event, and differences between patients with PNH⁺ and patients with PNH⁻ were assessed by the log-rank test. A paired *t* test analyzed changes in the proportions of PNH-type cells associated with IST. All statistical analyses were performed using JMP version 5.0.1J software (SAS Institute, Cary, NC).

# Results

## Validity of high-resolution flow cytometry

Figure 1B shows that a low-resolution assay using PE-labeled anti-CD55 and FITC-labeled anti-CD59 mAbs detected greater than 0.1% PNH-type RBCs in the peripheral blood of a healthy individual, whereas our assay of the same sample detected 0% PNH-type cells. Thus, the low-resolution assay could not discriminate a patient with AA with 0.1% PNH-type cells from a healthy individual, whereas our method revealed 0.04% PNH-type RBCs in the same patient, indicating a diagnosis of PNH⁺ AA. When the sensitivity of RBCs to complement-mediated lysis was examined using the modified Ham test, almost all RBCs in the glycophorin A⁺CD55⁻CD59⁻ fraction disappeared after an incubation in acidified saline containing human AB serum, verifying the reliability of our method for detecting PNH-type RBCs (Figure 1C). Conversely, when granulocytes from a patient with PNH⁺ AA were treated with aerolysin, approximately 99% of granulocytes in the CD11b⁺CD55⁺CD59⁺ fraction disappeared, whereas almost all cells in the CD11b⁺CD55⁻CD59⁻ fraction remained unchanged (Figure 1D), indicating that the few granulocytes in the CD11b⁺CD55⁻CD59⁻ fraction had the properties of PNH-type cells.
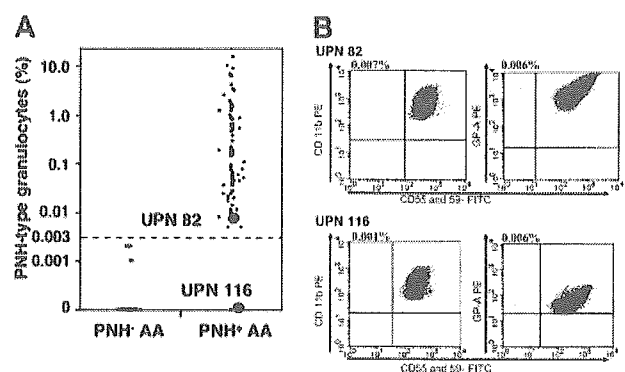
## Proportions of PNH-type cells in patients with AA

The proportion of PNH-type cells was increased in 83 (68%) patients. Among these patients with PNH⁺, the number of PNH-type cells was increased in both the granulocytes and RBCs of 69 (83%) of them, in only the granulocytes of 12 (15%), and in only the RBCs of 2 (2%). Figure 2A shows the proportions of PNH-type granulocytes and histograms from 2 patients with PNH⁺. Notably, the proportions of PNH-type granulocytes were below 0.1% in greater than 40% of patients with PNH⁺. Table 1 compares the clinical characteristics between patients with PNH⁺ and PNH⁻. Although the PNH⁺ group tended to be older and have higher WBC and MCV values than the PNH⁻ group, the clinical and hematologic parameters did not significantly differ between them.

## Response to ATG and CsA therapy

Sixty-eight of 83 (91%) patients with PNH⁺ improved with IST and achieved PR or CR at 12 months. However, only 18 of 39 (48%) patients with PNH⁻ responded to IST. Kaplan-Meier analysis showed that the chance of achieving PR was significantly better among patients with PNH⁺ than among patients with PNH⁻ (Figure 3A). The rate of obtaining CR at 5 years was also significantly higher in patients with PNH⁺ (36%) than in patients with PNH⁻ (3%) (Figure 3B). Multivariate analysis showed that among sex (male or female), age (older or younger than 40 years), severity (severe or moderate), presence or absence of chromosomal abnormalities, and presence or absence of increased PNH-type cells, only the presence of increased PNH-type granulocytes was a significant factor associated with good response to IST (*P* < .001). When patients with PNH⁺ were classified into 5 subgroups according to the proportions of PNH-type granulocytes (0.003%-0.01% in 7, 0.01%-0.1% in 21, 0.1%-1.0% in 22, 1.0%-10.0% in 13, 10.0%-23.1% in 3), the response rates to IST at 6 months did not significantly differ (88%, 74%, 90%, 81%, and 100%, respectively) among these subgroups. The responses of all of these subpopulations were significantly better than that of patients with PNH⁻.

## Prognosis after IST

The median follow-up period was 26.4 months (range, 0.1 to 71.4 months). In contrast to the response rates, the rates of overall survival at 5 years were comparable between patients with PNH⁺ (77%) and with PNH⁻ (71%) (Figure 4A). However, the probability of surviving failure free at 5 years was significantly higher in patients with PNH⁺ (64%) than in patients with PNH⁻ (12%) when



Figure 2. Proportions of PNH-type granulocytes. (A) Proportions of CD55⁻CD59⁻ granulocytes in each patient. (B) Histograms from one patient with PNH⁺ (UPN 82) with minimal PNH-type cells and from another patient with increased PNH-type cells only in RBCs (UPN 116).

**Table 1. Clinical characteristics of PNH⁺ and PNH⁻ patients**

| | PNH⁺ | PNH⁻ | P |
|---|---|---|---|
| No. of patients | 83 | 39 | NA |
| Median age, y (range) | 57 (13-83) | 54 (12-83) | .16 |
| Sex, M/F | 36/47 | 19/20 | .58 |
| Severity, severe/moderate | 53/30 | 22/17 | .43 |
| Chromosome abnormality, no. of patients | 7 | 3 | .88 |
| −7 | 0 | 1 | |
| +8 | 2 | 1 | |
| −Y | 3 | 0 | |
| Others | 2 | 1 | |
| Median WBC count, × 10⁹ (range) | 2.1 (0.5-4.3) | 1.9 (0.7-3.2) | .15 |
| Median neutrophil count, × 10⁹/L (range) | 0.53 (0.02-2.2) | 0.49 (0.01-2.7) | .65 |
| Median hemoglobin level, g/L (range) | 67 (32-140) | 67 (40-108) | .92 |
| Mean corpuscular volume, fL (range) | 101.5 (84.2-123.5) | 98.5 (77.2-118.0) | .13 |
| Median platelet count, × 10⁹/L (range) | 14.0 (2.0-60.0) | 16.0 (1.0-87.0) | .65 |
| Median reticulocyte count, × 10⁹/L (range) | 19.0 (3.0-90.0) | 24.0 (2.0-106.0) | .50 |
| Median time from diagnosis to IST, d (range) | 30 (1-334) | 33 (2-268) | .46 |
| No. of patients who received G-CSF during IST | 25 | 12 | .94 |

NA indicates not applicable.

failure-free survival was calculated based on time to treatment failure. This was defined as whichever came first among time from the first day of treatment until salvage treatment for nonresponse, relapse, development of a clonal hematologic disease (PNH, MDS, leukemia), solid tumor, or disease- or treatment-related death (Figure 4B). Although the probability of evolution into florid PNH or MDS at 5 years after IST did not significantly differ between patients with PNH⁺ (6% and 3%) and patients with PNH⁻ (0% and 4%) (Figure 4C), the probability of relapse tended to be higher in patients with PNH⁻ (36%) than in patients with PNH⁺ (21%) (Figure 4D). Two (2%) patients with PNH⁺ and 7 (18%) with PNH⁻ underwent allogeneic bone marrow transplantation (BMT) from related (n = 6) or unrelated (n = 3) donors because of failure to respond to IST (n = 6) and relapse of AA (n = 3). Rates of survival after BMT did not significantly differ between the 2 groups (data not shown).

### Changes in PNH-type granulocytes after IST

The presence of PNH-type cells after IST was serially tested in the peripheral blood of 53 of 122 patients. To characterize immune attack against hematopoietic stem cells that favors PNH-type cell clonal expansion, we examined the numbers of PNH-type cells in responsive patients. Figure 5A shows that the proportions of PNH-type granulocytes remained almost constant in 32 of 33 patients with PNH⁺ who responded to IST and decreased from 0.045% to 0% in only 1 patient (UPN 25). This indicates that the absolute number of PNH-type as well as of normal-type granulocytes increased in most responsive patients after IST. We compared the ratio of the degree of the increase in the absolute count between PNH-type (a) and normal-type (b) granulocytes before IST. The PNH-type granulocyte-to-normal-type granulocyte ratio in 32 patients ranged from 0.07 to 38.1 with a median of 1.06 (Figure 5B). The proportions of PNH-type cells did not change in 4 patients with PNH⁺ who were refractory to IST (Figure 5A-B). Sixteen patients with PNH⁻ were also tested after 6 to 24 months of IST. Only one patient who had achieved PR became PNH⁺ at 24 months and then relapsed with AA at 29 months after IST.

The proportions of PNH-type granulocytes were repeatedly determined in 23 patients for more than 24 months after IST. Figure 5C shows that the proportions remained constant over a long period in most patients including one (UPN 106) who had 0.1% PNH-type granulocytes (Figure 5D). The proportion of PNH-type granulo-

cytes significantly increased from 3.31% to 76.0% in only one patient during the 4-year observation period.

## Discussion

An increase in the proportion of PNH-type cells in peripheral blood has been implicated in the immune pathophysiology of bone marrow failure.[10] Several studies including our previous investigation found a correlation between an increase in the proportion of PNH-type cells and a favorable response to IST among patients with MDS[11,12,15] and with AA.[16,26] However, the clinical application of these findings has been hampered. Small patient cohorts and the relatively low prevalence of an increased number of PNH-type cells in these studies have led to concerns about unreliability of the correlation. The present study based on a larger number of patients with recently diagnosed AA conclusively demonstrated that a minor population of PNH-type cells predicts a good response to IST as well as good prognosis for patients with AA after IST.
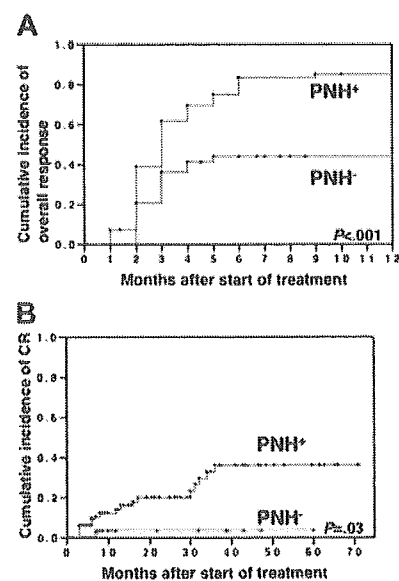


Figure 3. Response to immunosuppressive therapy. Incidence of overall (A) and complete (B) responses in patients with PNH⁺ and PNH⁻.
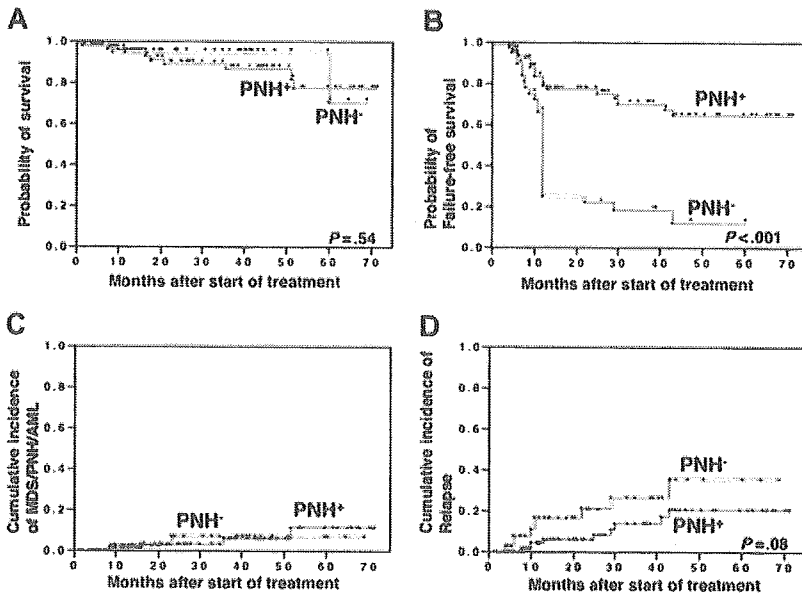
Figure 4. Prognosis after IST compared between patients with PNH+ and with PNH-. (A) Overall survival; (B) failure-free survival; (C) incidence of clonal hematologic disorders, including PNH, myelodysplastic syndrome, and acute myelogenous leukemia; and (D) incidence of relapse.

The reliability of our high-resolution flow cytometry, which was verified by the modified Ham test and by aerolysin treatment, revealed an increase in the number of PNH-type cells in 68% of the patients with AA. This was considerably higher than the reported prevalence.

The clinical features and overall survival rates did not significantly differ between patients with PNH+ and patients with PNH- in the present study. However, failure-free survival was obviously better among patients with PNH+ than patients with PNH-. This indicated that, although patients with PNH- can survive as long as

patients with PNH+ after IST, they often require salvage or supportive treatment such as allogeneic stem cell transplantation and blood transfusions, because of a partial response to IST or a high rate of relapse. Contrary to the expectation based on the presence of abnormal hematopoietic clones such as PNH-type cells, the probability of evolving into clinical PNH or MDS in patients with PNH+ was comparable to that in patients with PNH-. The proportions of PNH-type granulocytes remained stable over a period of 1 to 66 months in most patients with PNH+, a finding consistent with previous reports.[26,27] These findings indicate that
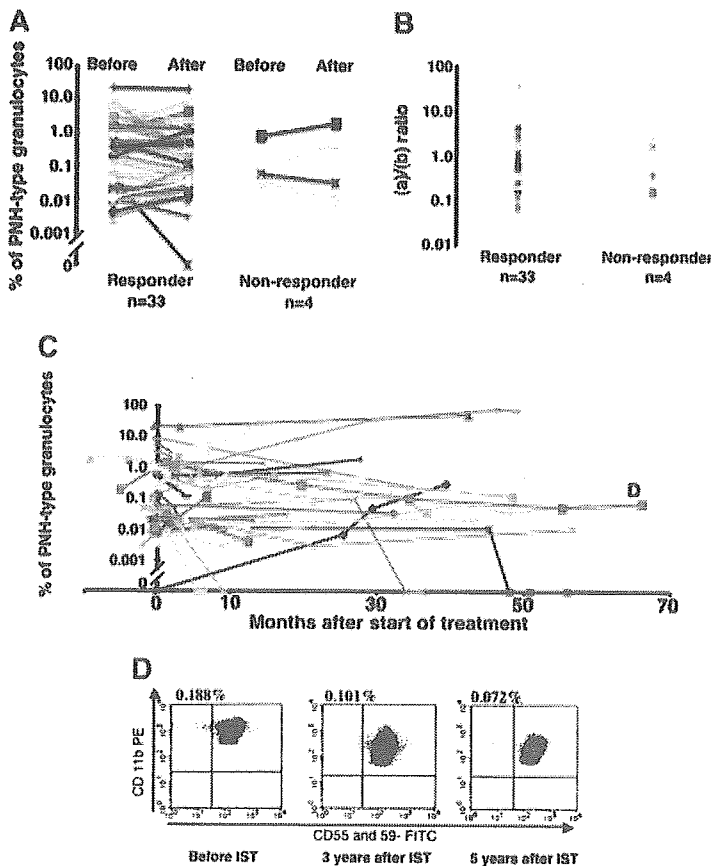


Figure 5. Changes in proportions of PNH-type granulocytes associated with responses to IST. (A) Change in responders and nonresponders. (B) Proportions of granulocyte counts after and before IST determined for PNH-type (a) and normal-type (b) granulocytes and ratios of PNH-type granulocytes (a) to normal-type granulocytes (b) were plotted. (C) Longitudinal analysis of PNH-type granulocytes. Proportions of PNH-type granulocytes of 37 patients with PNH+ and 1 patient with PNH- who became PNH+ (black line) were displayed. (D) Changes in proportions of PNH-type granulocytes over 5 years in patient UPN 106 with AA (shown as D in Figure 5C).

the presence of an increased proportion of PNH-type cells predicts not only a positive response but also a good quality of response to IST among patients with AA.

The significantly high response rate to IST among patients with PNH$^+$ AA suggests that PNH$^+$ AA is an authentic type of immune-mediated marrow failure. In line with this hypothesis, patients with PNH$^+$ AA often have a specific HLA-DR allele (HLA-DR15) and antigen-driven T-cell proliferation in the bone marrow.[12,28] Furthermore, antibodies against diazepam-binding inhibitor-related sequence-1 (DRS-1), a peroxisomal protein abundantly expressed by hematopoietic progenitor cells, are frequently detected in sera from patients with PNH$^+$ AA.[29] However, the relatively low response rate to IST among patients with PNH$^-$ AA indicates that a heterogeneous pathophysiology might underlie this subset of AA. In line with this notion as described in our previous study,[16] clonal hematopoiesis arose more frequently in patients with PNH$^-$ AA than in patients with PNH$^+$ AA. Even among patients who responded to IST, patients with PNH$^-$ AA rarely achieved complete recovery of hematopoiesis and were susceptible to AA relapse. Immune mechanisms that are not associated with an increase in the proportion of PNH-type cells might damage hematopoietic stem cells more profoundly than those in PNH$^+$ AA.

PNH-type stem cells might acquire a survival advantage over normal-type stem cells when T or natural killer (NK) cells attack hematopoietic stem cells.[30-32] The high response rate to IST in patients with PNH$^+$ AA indicates that such an immune mechanism is functional in this subset of AA. If the immune mechanisms were responsible for bone marrow failure, IST would more efficiently induce expansion of normal-type than of PNH-type stem cells. However, in most patients with PNH$^+$, successful IST resulted in a similar increase in the number of both PNH-type and normal-type

granulocytes, which contradicts the immune escape theory. A similar finding has been reported by Maciejewski et al[26] for patients with AA with 1% or more CD15$^+$CD66b$^-$CD16$^-$ granulocytes. One possible explanation for this discrepancy is as follows. An immune attack against hematopoietic stem cells at the onset of AA that allows PNH-type stem cells to survive does not contribute to the subsequent progression of bone marrow failure, which is caused by different immune mechanisms targeting epitopes other than those that induce disease. Such epitope spreading occurs in the development of other immune diseases such as multiple sclerosis.[33] Alternatively, the suppression of hematopoiesis after the clonal expansion of PNH-type cells might be caused by myelosuppressive cytokines rather than antigen-specific T cells.

The presence of a few PNH-type cells has profound significance for the management of patients with recently diagnosed AA. Although those who have PNH$^-$ AA can improve with IST, the maximal response rate is 50% and the rate of failure-free survival at 5 years is below 20%. Therefore, allogeneic BMT is recommended more often than IST for young patients with PNH$^-$ who have HLA-compatible sibling donors. Conversely, IST is more frequently recommended than BMT for patients with PNH$^+$, particularly when the likelihood of BMT-related mortality is high. Among patients with AA who are unresponsive to the initial ATG and CsA therapy, those who benefit from a second IST might be PNH$^+$. Conventional flow cytometry capable of detecting 1% or more PNH-type cells would also be clinically useful in predicting response to IST because the response to IST does not change according to the proportion of PNH-type cells. The predictive value of an increased proportion of PNH-type cells for a favorable prognosis in AA identified here warrants a further worldwide prospective study on non-Japanese patients with AA.

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# STEM CELLS®

# Roles of Stat3 and ERK in G-CSF Signaling

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## ABSTRACT

G-CSF specifically stimulates the proliferation and differentiation of cells that are committed to the neutrophil-granulocyte lineage. Although Stat3 was thought to be essential for the transduction of G-CSF–induced cell proliferation and differentiation signals, mice deficient for Stat3 in hematopoietic cells show neutrocytosis and infiltration of cells into the digestive tract. The number of progenitor cells in the neutrophil lineage is not changed, and G-CSF–induced proliferation of progenitor cells and prolonged neutrophil survival were observed in Stat3-deficient mice. In hematopoietic cells from Stat3-deficient mice, trace levels of SOCS3, a negative regulator of granulopoiesis, were observed, and SOCS3 expression was not induced by G-CSF stimulation. Stat3-null bone marrow cells displayed a significant activation of extracellular regulated kinase 1 (ERK1)/ERK2 under basal conditions, and the activation of ERK was enhanced and sustained by G-CSF stimulation. Furthermore, the augmented proliferation of Stat3-deficient bone marrow cells in response to G-CSF was dramatically decreased by addition of a MEK1 inhibitor. These results indicate that Stat3 functions as a negative regulator of G-CSF signaling by inducing SOCS3 expression and that ERK activation is the major factor responsible for inducing the proliferation of hematopoietic cells in response to G-CSF. STEM CELLS 2005;23:252–263

## INTRODUCTION

The proliferation and differentiation of hematopoietic precursor cells are regulated by a family of cytokines. In particular, granulocyte colony-stimulating factor (G-CSF) specifically stimulates the proliferation and differentiation of cells that are committed to the neutrophil lineage [1]. The biological functions of G-CSF are mediated through binding to a cell-surface receptor that is predominantly expressed on neutrophilic progenitor cells and mature neutrophilic granulocytes, although the receptor is also expressed on hematopoietic progenitor cells [2]. The binding of G-CSF to its receptor induces the tyrosine phosphorylation of Jak1, Jak2, and Tyk2 [3–6], which are members of the Janus family of protein tyrosine kinases (Jaks) [7]. Activated Jaks phosphorylate residues in the cytosolic

tails of G-CSF receptors, allowing subsequent recruitment of various signaling proteins to the receptor complex. Members of the signal transducers and activators of transcription (Stat) family are recruited and phosphorylated by Jak kinase, translocate to the nucleus, and bind the promoter regions of target genes [8, 9]. G-CSF stimulation results in the specific phosphorylation of Stat3 [4, 10] and, more infrequently, Stat5 and Stat1 [6, 11]. Generally, this Jak-Stat signaling pathway is thought to be essential for the transduction of cytokine signaling [12], but other cytoplasmic protein tyrosine kinases, including lyn and syk [13, 14], are also phosphorylated and activated in response to G-CSF signaling.

The role of Jak kinases in the G-CSF signaling pathway was initially examined by the use of cell lines that were deficient for each of the Jak kinases. G-CSF induces the tyrosine phosphorylation and activation of Jak1, Jak2, and Tyk2, and the absence of one Jak does not preclude G-CSF–induced tyrosine phosphorylation of the remaining Jaks. However, in the absence of Jak1, G-CSF does not induce receptor tyrosine phosphorylation, and the induced tyrosine phosphorylation of Stat proteins is greatly reduced [6]. Although multiple Jaks are activated by G-CSF, Jak1 is in a unique position to phosphorylate the receptor and thereby affect Stat protein tyrosine phosphorylation because of either location within the receptor complex or substrate specificity. However, Jak1-deficient mice do not show neutropenia, and Jak1 deficiency has no effect on the colony formation of bone marrow cells induced by G-CSF [15]. Furthermore, deletion of either Jak2 or Tyk2 has no effect on G-CSF–induced colony formation of bone marrow cells [16, 17]. These results indicate that there is redundancy among the Jak kinases in G-CSF signaling, and the lack of one Jak kinase may be compensated for by the activation of other Jak kinases. Interestingly, the involvement of Jak kinases in G-CSF signaling seems to be different from that in other cytokine-signaling pathways. For example, Jak1 is essential for gp130, interferon (IFN), and interleukin (IL-7)–mediated signaling [15], Jak2 is essential for erythropoietin (Epo) signaling [17], Jak3 is essential for IL-2 receptor common gamma chain–mediated signaling [15], and Tyk2 is essential for IL-12 signaling [16].

In addition, we and others have used Stat-deficient mice to demonstrate that Stats play an essential and nonredundant role in cytokine signaling [18–22]. G-CSF stimulation activates mainly Stat3 and, to a lesser extent, Stat1 and Stat5 in bone marrow cells. Stat1 and Stat5a/b-deficient mice have normal neutrophil numbers, although colony formation by bone marrow cells in response to G-CSF stimulation is decreased by the absence of Stat5a/b [18, 21]. However, the expression of dominant-negative Stat3 in 32Dcl3 cells, which differentiate into neutrophils after G-CSF treatment, does not prevent them from proliferating in response to G-CSF

[23]. Additionally, transgenic mice with a targeted mutation of the G-CSF receptor that abolishes G-CSF–dependent Stat3 activation show severe neutropenia, with an accumulation of immature myeloid precursors in the bone marrow [24]; constitutively active Stat3 partially rescues this neutropenia. Stat3 is then thought to transduce the proliferation and differentiation signal of G-CSF. Ablation of Stat3 produced early embryonic lethality [25], and selective ablation of Stat3 in neutrophils and monocytes by Cre recombinase-dependent gene deletion directed by the macrophage lysozyme promoter did not affect neutrophil production, suggesting that Stat3 is required at an early stage of neutrophil development. To clarify the role of Stat3 in the G-CSF signaling pathway, we have examined the role of Stat3 in hematopoiesis by selective ablation of the *Stat3* gene in hematopoietic progenitor cells. In contrast to expectations, mice that were deficient for *Stat3* in hematopoietic cells show neutrocytosis and infiltration of cells into the digestive tract.

## MATERIALS AND METHODS

### Mice

Stat3$^{flox/-}$ mice were generated by mating Stat3$^{flox/+}$ mice, in which the DNA base pairs encoding the tyrosine phosphorylation site in Stat3 are flanked by two loxP sites (Stat3$^{flox/flox}$) [26], with Stat3$^{+/-}$ mice, in which exons 20 through 22 are replaced by a neomycin resistance gene in the knockout allele [27]. To establish mice with a conditional knockout of Stat3 in hematopoietic cells, Stat3$^{flox/-}$ mice were mated with a transgenic line bearing Cre recombinase driven by the IFN-inducible Mx promoter [28]. Genotyping was performed by polymerase chain reaction analysis of genomic tail DNA. The primer sequences were as follows: Stat3-flox: 5'-cctgaagac-caagttcatctgtgtgac-3' and 5'-cacacaagccatcaaactctggtctcc-3'; Stat3⁻: 5'-agcagctgacaacgctggctgagaagct-3' and 5'-atcgccttc-tatcgccttcttgacgag-3'; Mx1-Cre: 5'-ggacatgttcagggatcgc-caggcg-3' and 5'-gcataaccagtgaaacagcattgctg-3'. Expression of Cre was induced by injecting mice intraperitoneally with 250 µg of polyinocinic-polycytidylic acid (pIpC) (Sigma, St. Louis) three times at 2-day intervals as previously described [28]. Age-matched Stat3$^{flox/-}$ and Mx1-Cre:Stat3$^{flox/-}$ were injected with pIpC and used for further experiments 2 weeks later, except where noted in the text. Mice were housed and bred in the Kyushu University Animal Center.

### Histological and Hematological Analysis

Tissues were fixed in 10% phosphate-buffered formalin, and paraffin-embedded tissue sections were stained with hematoxylin and eosin using standard techniques.

Complete blood counts were analyzed using Celltac α (Nihon Kohden, Tokyo). Peripheral and bone marrow blood

cells were prepared on slide glasses and stained with Giemsa solution. Differential cell counts were scored visually.

## Preparation of Neutrophils from the Peritoneal Cavity

Mice were injected intraperitoneally with 2 ml of 4% thioglycollate. After 4 hours, peritoneal neutrophils were obtained by peritoneal lavage with 10 ml of ice-cold phosphate-buffered saline (PBS). After an 8-hour incubation in plastic dishes, nonadherent cells were harvested and used for further experiments. These cells were >90% Gr-1$^+$, as determined by flow cytometry.

## Evaluation of Apoptosis

To evaluate apoptosis, cells were incubated with fluorescein isothiocyanate (FITC)–conjugated Gr1 (Becton, Dickinson, San Jose, CA) and propidium iodide for 30 minutes at 4°C, washed twice in PBS, and analyzed on a FACS Calibur (Becton, Dickinson). To exclude the dead cells, the gated nucleated cells were used for further examination.

## Chemotaxis Assay

The neutrophil chemotaxis assay was performed as described [29]. Briefly, the lower well contained 800 µl of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS) and $1 \times 10^{-7}$ M formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma), and the upper chemotaxicell (Kurabo, Osaka) chamber contained 400 µl of a neutrophil suspension. After incubation for 1 hour at 37°C, the cells that had passed through the membrane were counted.

## Hematopoietic Progenitor Cell Assays

The frequency of hematopoietic progenitor cells was determined by clonogenic assays in methylcellulose, as described previously [30]. Briefly, bone marrow or peripheral blood mononuclear cells were separated by Ficoll-Paque sedimentation, and $1 \times 10^5$ or $2 \times 10^5$ cells were cultured in methylcellulose containing IL-3 (20 ng/ml), stem cell factor (SCF) (20 ng/ml), and Epo (4 U/ml) for the colony-forming unit-granulocyte, macrophage (CFU-GM), BFU-E, and CFU-culture (CFU-C) assays or G-CSF (50 ng/ml) only for the CFU-G assay. CFU-GM, CFU-G, and BFU-E were measured after 10 days in culture.

## In Vitro Proliferation Assays

Bone marrow mononuclear cells ($1 \times 10^6$/mi) were incubated for 3 days in IMDM supplemented with 30% FCS in the presence or absence of IL-3 (1 ng/ml) and G-CSF (10 ng/ml). To assess the effects of an MEK inhibitor, dimethyl sulfoxide (DMSO) or U0126 (Cell Signaling, Beverly, MA) dissolved in DMSO was added. After 72 hours, 0.5 µCi $^3$H-thymidine

was added and the cells were incubated for an additional 8 hours. Proliferative activity was determined by $^3$H-thymidine incorporation.

## In Vivo Administration of G-CSF or Thrombopoietin

Mice were injected subcutaneously with either G-CSF (50 µg/kg daily for the indicated period) or with thrombopoietin (TPO) (30 µg/kg for 5 days). Peripheral blood was collected on the indicated day.

## Dextran Sulfate Sodium–Induced Colitis (DSS)

Colitis was induced by feeding mice drinking water supplemented with 4% DSS (Wako, Osaka, Japan), as described previously [31]. Control mice were fed drinking water without DSS.

## Western Blotting

Bone marrow cells were treated with G-CSF for the indicated time and were then lysed in lysis buffer as previously described [32]. Cell lysates were centrifuged at 12,000$g$ for 15 minutes to remove debris. Total cell lysates were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed using the indicated antibodies and visualized with an ECL detection system (Amersham, Uppsala, Sweden). Anti-phospho-ERK1/2, anti-phospho-p38, anti-ERK2, anti-phospho-Stat3, and anti-phospho-Stat5 antibodies were purchased from Cell Signaling. Anti-Stat3 and anti-Stat5 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-SOCS3 antibody was purchased from IBL (Gunma, Japan).

## RESULTS

### Increased Numbers of Circulating Neutrophils and Myeloid Cells in the Bone Marrow in Stat3-Deficient Mice

To determine whether Stat3 plays a role in hematopoiesis, we used the Cre-loxP recombination system. To decrease the amount of residual Stat3 protein after Cre-mediated deletion, we crossed Stat3$^{flox/-}$ mice with a transgenic line bearing Cre recombinase driven by the IFN-inducible Mx promoter. Only trace amounts of Stat3 were detected after induction of Mx-Cre by treatment with pIpC (Fig. 1A). When bone marrow cells from pIpC-treated Mx1:Stat3$^{flox/-}$ mice were treated with G-CSF, the phosphorylation of Stat3 was diminished, whereas Stat5 was phosphorylated at the same extent as wild-type mice by IL-3 stimulation.

We next examined the role of Stat3 in granulopoiesis in vivo. Blood cell counts from wild-type and Mx1:Stat3$^{flox/-}$

**Table 1.** Complete blood counts

| | Stat3[flox/-] | Mx1:Stat3[flox/-] |
|---|---|---|
| White blood cell ($\times 10^3$/μL) | 6.64 ± 2.23 | 7.84 ± 2.57 |
| Neutrophil (per μL) | 1,800 ± 953 | 4,172 ± 1,889 |
| Lymphocyte (per μL) | 3,868 ± 1,552 | 2,787 ± 1,009 |
| Monocyte (per μL) | 664 ± 359 | 522 ± 295 |
| Eosinophil (per μL) | 256 ± 225 | 324 ± 267 |
| Hematocrit (%) | 47.4 ± 2.2 | 45.7 ± 2.6 |
| Platelet ($\times 10^4$/μL) | 80.7 ± 27.6 | 75.1 ± 22.4 |

Peripheral blood count analyses from polyinocinic-polycytidylic acid–treated Stat3[flox/-] and Mx1:Stat3[flox/-] mice ($n = 18$) at ages of 7–10 weeks. White blood cell differential counts were examined by Giemsa staining.

littermates 2 weeks after deletion of Stat3 are shown in Table 1. Morphologically identifiable neutrophils were detected in the peripheral blood of mice lacking Stat3 in hematopoietic cells, and the number of circulating neutrophils in Mx1:Stat3[flox/-] mice was 2.3-fold greater than in control mice. There were no differences in the numbers of lymphocytes, erythrocytes, or platelets due to ablation of Stat3. In bone marrow, the number of mature myeloid cells was greater in Mx1:Stat3[flox/-] mice than in control littermates, which is consistent with the observations in peripheral blood (Fig. 1B).

## Effects of Stat3 Deletion on the Frequency of Myeloid and Erythroid Progenitor Cells and on the Response to G-CSF in Bone Marrow Cells

Because the number of neutrophils was higher in the Mx1: Stat3[flox/-] mice, we next determined the number of hematopoietic progenitor cells in the bone marrow. Bone marrow from pIpC-treated control or Mx1:Stat3[flox/-] mice was seeded in methylcellulose containing IL-3, SCF, and Epo to determine CFU-GM or in methylcellulose containing G-CSF to determine CFU-G. The CFU-GM and BFU-E cloning efficiencies were comparable in Stat3[flox/-] and Mx1:Stat3[flox/-] bone marrow cells in the presence of IL-3, SCF, and Epo (Fig. 2A). Similarly, the total colony numbers were not altered due to the absence of Stat3 (data not shown). The number of CFU-G induced by G-CSF was also unaffected by the absence of Stat3 (Fig. 2A).

Because the number of myeloid progenitor cells was not affected by the absence of Stat3, we next examined the effect of Stat3 deletion in bone marrow cells for the proliferation by cytokine stimulation. Bone marrow cells taken from Mx1:Stat3[flox/-] mice and Stat3[flox/-] mice 14 days after pIpC treatment responded to IL-3 to a similar extent. In contrast, the proliferation of bone marrow cells after G-CSF stimulation was enhanced in Mx1:Stat3[flox/-] mice 14 days after pIpC treatment compared with control littermates (Fig. 2B).

To examine the possibility that Stat3 is involved in G-CSF–mediated cell survival, we determined

the susceptibility of neutrophils to apoptosis in the presence of 10 ng/ml of G-CSF (Fig. 2C). After 48 hours in media supplemented with G-CSF, there was
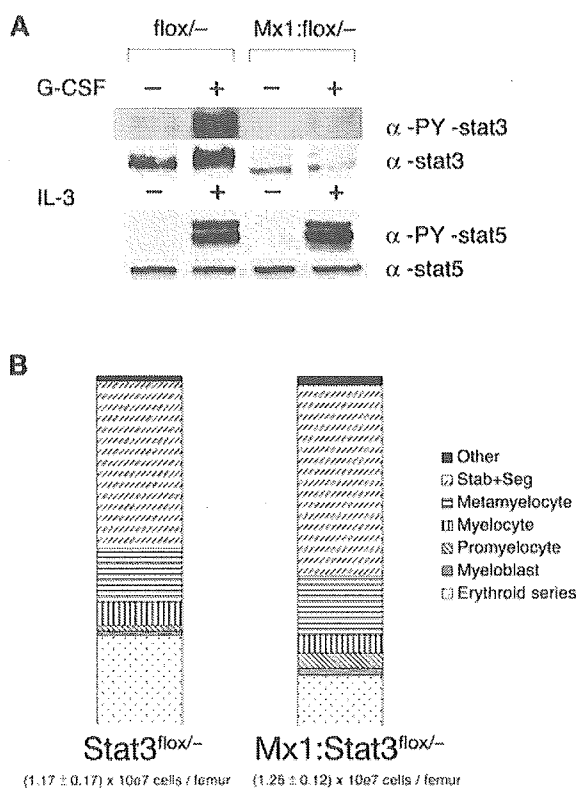


**Figure 1. (A):** Phosphorylation of Stat3 or Stat5 in response to G-CSF or IL-3 stimulation of bone marrow cells from Stat3[flox/-] and Mx1:Stat3[flox/-] mice. Bone marrow cells from Stat3[flox/-] and Mx1:Stat3[flox/-] mice were incubated for 8 hours in the absence of cytokines and then stimulated with G-CSF (50 ng/ml) or IL-3 (10 ng/ml) for 30 minutes. Total cell lysates were analyzed by Western blot with the indicated antibodies. **(B):** Bone marrow analysis from Stat3[flox/-] and Mx1:Stat3[flox/-] mice. Bone marrow differential counts were performed on preparations from Stat3[flox/-] and Mx1:Stat3[flox/-] mice that were euthanized 2 weeks after treatment with polyinocinic-polycytidylic acid. Abbreviation: IL, interleukin.